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Studies on the Binding of Bilirubin to Albumin

by

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A thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy (Ph.D) of the
University of Warwick.

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Preface

The work described in this thesis was carried out in the Department of Chemistry and Molecular Sciences, University of Warwick, Coventry, England, during the period between October, 1977 and August, 1980.

It is the original work of the author, except where specific acknowledgement is made or implied. This thesis has been submitted at the University of Warwick alone, in fulfilment of the requirements for the degree of Ph.D.

'With a little help from my friends'

The Beatles, 1966

Acknowledgements

I am grateful to all those people who, have, in their various ways, made this thesis possible. More specifically I owe a special debt of gratitude to the United Nations for financial support. I also express my gratitude to the chairman and staff of Chemistry and Molecular Sciences for permission to use their research facilities throughout this work.

The largest single debt I owe to Dr. D. W. Hutchinson, who has constantly succeeded in creating a unique environment for research, characterised alike by friendliness, by scientific enthusiasm, and by helpful supervision and encouragement. I am also indebted to the following: Mr. B. W. Augustus, Dr. M. A. Benhra and Mr. C. F. Hui for their useful discussions, or otherwise.

My sincere thanks are due to Thelma and little Sphiwo, in appreciation of their unlimited patience and forbearance to enable me to complete this work.

Finally I am grateful to Barbara Painter for help in typing this thesis.

Summary

In this thesis, the work undertaken in an attempt to gain insight into the location of the binding site of bilirubin is described.

The history, properties and biosynthesis of both bilirubin and albumin are reviewed in Chapter One. Chapter Two contains an account of the novel one-step method of labelling bilirubin of high yield and specific activity. In Chapter Three, the effect of light on bilirubin in the presence and absence of albumin, followed by the labelling of the albumin in an attempt to obtain information regarding the nature of the amino acid residues involved in the binding of bilirubin to albumin. Attachment of labelled bilirubin to albumin enabled the possible site on the latter to be followed during degrading procedures. Possible effect of this photo-induced binding on phototherapy is described.

Chapter Four contains an account of the synthesis and binding studies of mesobilirubin. Fluorescence and spectroscopic methods are employed to determine the association constants of mesobilirubin and comparison with bilirubin made. The importance of the vinyl groups in bilirubin binding is cited and the possibility of mesobilirubin being a kernicteric pigment is hypothesized.

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Abbreviations

The following abbreviations are used in the text.

ALA	δ - aminolaevulinic acid
NADPH	Reduced nicotinamide adenine dinucleotide phosphate.
HSA	Human serum albumin
EDTA	Ethylenediamine tetraacetic acid
DMSO	Dimethyl sulphoxide
m.p.	melting point
ELB	early labelled bilirubin
CD	circular dichroism
THF	Tetrahydrofuran
TLC	Thin layer chromatography
PLC	Preparative layer chromatography

The following abbreviations are used in the diagrams

M	CH_3
V	$\text{CH} = \text{CH}_2$
Et	$\text{CH}_2 \text{CH}_3$
P	$\text{CH}_2 \text{CH}_2 \text{COOH}$

Notes on Experimental Section

Ultra violet and absorption spectra (u.v.) were recorded with either a Unicam SP800 or Cecil 505 spectrophotometer. Wavelengths are recorded in nm , calibrated against a holmium filter. Extinction coefficients were determined on a Unicam SP500 Spectrophotometer.

Melting points (m.p.) were determined using a Reichert heated microscope stage and are uncorrected.

Thin Layer chromatography (TLC) and preparative layer chromatography (PLC) were carried out on silica gel PF 254 (U.V. sensitive) at thickness 0.5 mm and 1.0 mm respectively and on 20 x 20 cm plates.

Specific activities of radioactive samples were determined using a PRLAS scintillation counter in Toluene (600mL), Ethoxyethanol (400mL), PPO (4g) and POPOP (0.2g) cocktail.

CHAPTER 1

1. General Introduction

1:1 Historical Background

The history of the bile pigments dates as far back as the second century A.D. when Galen associated jaundice with the presence of stones in the biliary tract. However, not until 1847, did Virchow demonstrate the association of bile pigment with haemoglobin.¹ He described the formation of bilirubin crystals (haematoidin) in blood extravasates. Tarchanoff 1874² showed that haemoglobin was converted to bilirubin. This then became the basis of theories on the mechanism of conversion of haemoglobin to bilirubin. Having synthesized haemin³ and bilirubin⁴, Hans Fischer was able to show that bilirubin was formed from haemin.⁵

1:2 Structure and Biosynthesis of Bile Pigments

The bile pigments or bilinoids are open chain tetrapyrroles with the skeletal structure 3 (Figure 1). This basic structure is called bilin; by convention, (i) in the absence of specific information on the imino hydrogen location, the 22-H tautomer is drawn, and (ii) number 20 is omitted; C-20 is a phantom atom that corresponds to the extra carbon atom that would be required to transform the bilinoid into a porphyrin ring (structure 1, Figure 1). Naturally occurring bilinoids have oxygen at the terminal positions, and are formally 1, 19 - dihydroxy derivatives of bilin or 10, 23 - dihydrobilin. As the natural compounds appear to be predominantly in the tautomeric bislactam form, they are commonly depicted in that form, but for convenience denoted by the dihydroxy names.⁶

Biliverdin, mesobilirubin including bilirubin (structure 4, Figure 1) are trivial names used to denote specific bile pigments. 1, 19 -

Dihydroxy derivatives of 10, 23 - dihydrobilin are commonly called "(bili)mbins", and 1, 19 - dihydroxy derivatives of bilin called "(bili)verdins". The configuration of β - substituents in rubins and verdins is denoted by reference to the corresponding protoporphyrin isomer that has the same sequence of substituents. Thus, the trivial name is followed by a Roman numeral (e.g. IX), corresponding to that used to designate the isomeric type of the precursor porphyrin, and by a Greek letter (e.g. α), indicating which one of the porphyrin meso-bridges (α, β, γ or δ) corresponds to the phantom C-20 carbon atom of the bilinoid. Following this nomenclature, bilirubin - IX α (structure 4) corresponds to the rubin that is formed by cleavage of protoporphyrin- IX (structure 2) at the α meso bridge (Figure 1). Conventionally, however, the term "bilirubin" is used to specifically denote bilirubin - IX α .

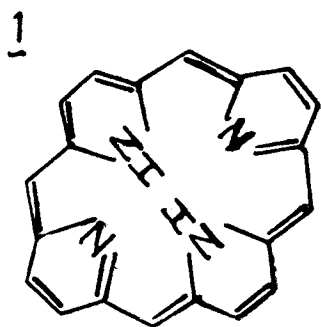
According to the sequence of the β - substituents on the pyrrole rings (positions 2, 3, 7, 8, 12, 13, 17, 18) several isomeric types of porphyrins with the same elementary composition exist, and a "type" nomenclature, using Roman numerals, is used to distinguish these isomers. The protoporphyrin ring by convention corresponds to protoporphyrin - IX (structure 2 in Figure 1). The nature and sequence of the other β - substituents of porphyrins and the trivial name and type designation of the corresponding compound, is shown in Table 1.

Depending on the configuration (Z or E) at the C-5, C-10 and C-15 methene bridges, several geometrical isomers of bilins exist. Thus, bilirubin can occur in the form of four geometrical isomers (Figure 1): $4Z, 15Z$ - bilirubin (structure 4), $4E, 15Z$ - bilirubin (structure 5), $4Z, 15E$ - bilirubin (not shown), and $4E, 15E$ - bilirubin (structure 6).

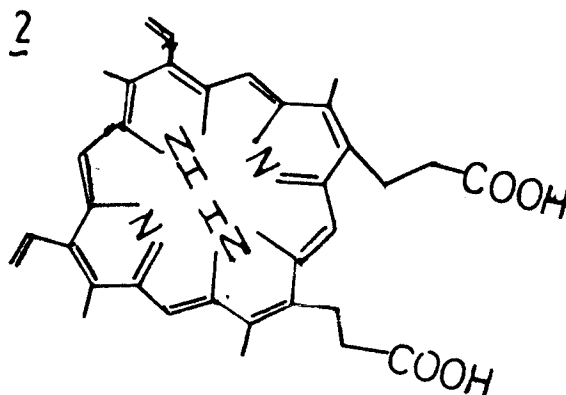
This thesis will concentrate on bilirubin - IX α and the other members of the series are bilirubin XIII α and III α and mesobilirubin

Figure 1

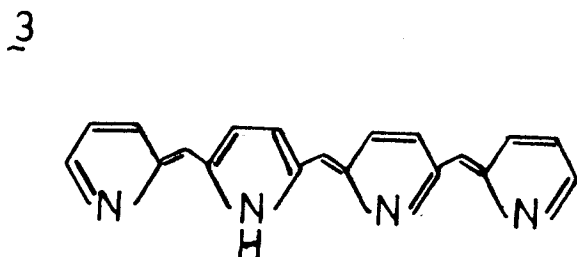
Structures of Porphyrins and Bilinoids



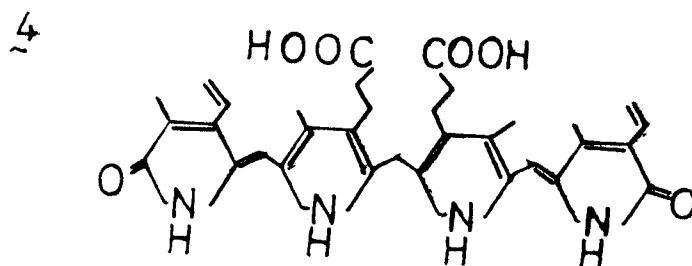
PORPHYRIN



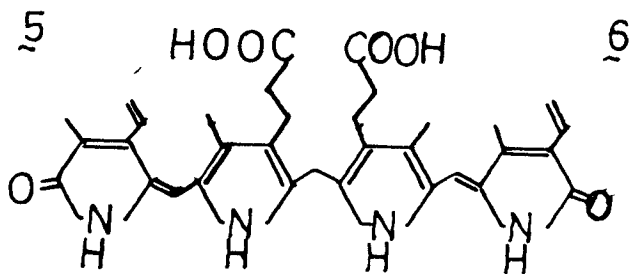
PROTOPORPHYRIN IX



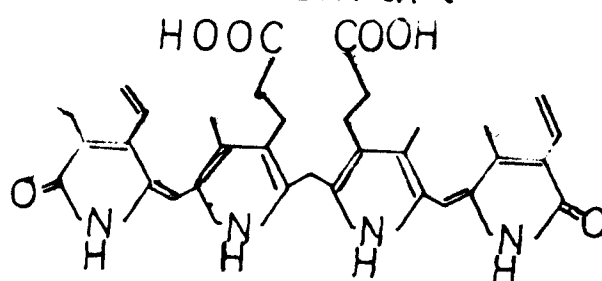
BILIN



5Z,15Z BILIRUBIN IX α



5E,15Z BILIRUBIN IX α



5E,15E BILIRUBIN IX α

Trivial Names of Some Important Porphyrins Substituents at positions

Name	2	3	7	8	12	13	17	18
Protoporphyrin - IX	Me	V	Me	P	P	Me	Me	V
Protoporphyrin - III	V	Me	Me	P	P	Me	Me	V
Protoporphyrin - XIII	Me	V	Me	P	P	Me	V	Me
Uroporphyrin - I	A	P	A	P	A	P	A	P
Uroporphyrin - III	A	P	A	P	P	A	A	P
Coproporphyrin - III	Me	P	Me	P	P	Me	Me	P
Mesoporphyrin - IX	Me	Et	Me	P	P	Me	Me	Et

IX α , XIII α and III α , which are fully discussed elsewhere.^{7,8}

Bilirubin (Figure 1) is an open tetrapyrrole, principally formed as a degradation product of ferroprotoporphyrin - IX (heme). This molecule is an iron - containing tetrapyrrole macrocycle and plays a critical role in aerobic metabolism by reversibly binding oxygen in haemoglobin and myoglobin, and by serving as the active site in detoxification reactions catalysed by hemoprotein enzymes.⁶ Heme may also have important regulatory functions in protein synthesis. Important cyclic tetrapyrroles in nature related to heme are chlorophylls, which contain magnesium and were found^{9,10} to be derived from protoporphyrin - IX, and vitamin B₁₂, a corrinoid^{11, 12} derived from uroporphyrinogen - III (Figure 2).

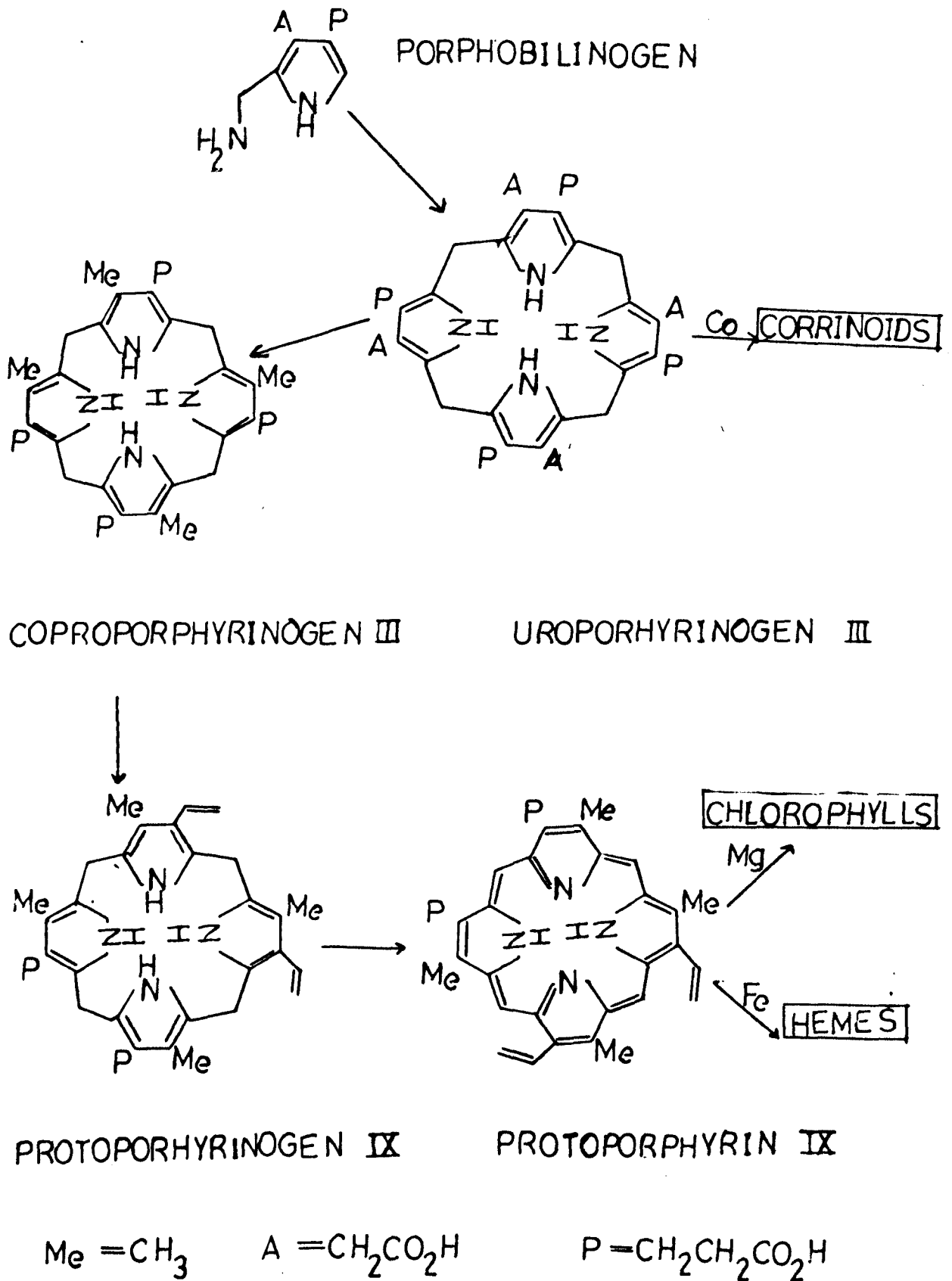
Whereas open tetrapyrroles have an important physiological role in algae by serving as prosthetic group of the photosynthetic biliproteins, bilirubin in mammals merely is a waste product without any obvious function. However, the association of this pigment with yellow jaundice has interested, clinicians and investigators. Bilirubin, therefore, a potentially neurotoxic compound causes bilirubin encephalopathy and psychomotoric retardation in neonates with severe hyperbilirubinemia. These cytotoxic effects depend on its binding to intra - and extracellular "carrier" proteins and conversion into polar glycosides. This configuration is catalyzed by microsomal UDP - glucuronyltransferase, an enzyme system that also plays a key role in detoxification and disposition of many other endogenous metabolites and xenobiotics.

Bilirubin is formed by cleavage of the heme macrocycle almost exclusively at the α -methene bridge so that virtually all the bilirubin formed has the IX α configuration. This bilirubin formation therefore reflects the continuing turnover of heme and essential hemoproteins such as haemoglobin, myoglobin, cytochromes and other hemoprotein enzymes.

The daily production in normal adults estimated by radiolabelled

Figure 2

Biosynthesis of Hemes, chlorophylls and Corrinoids



bilirubin¹³ or by measurements of endogenous carbon monoxide production^{14,15} averages 250-350 mg per animal. Calculations of bilirubin production from carbon monoxide exhalation is based on the assumption that in normal individuals all heme degradation occurs by oxidative cleavage at a methene bridge and thus yields equimolar amounts of carbon monoxide and bilirubin. Carbon monoxide production, therefore, probably reflects total heme turnover in the body, whereas plasma bilirubin turnover reflects only the pigment fraction that enters the plasma prior to excretion and hence fails to account for bilirubin that is formed in the liver and directly excreted into bile.¹⁶ These considerations probably explain why studies of plasma bilirubin turnover yield values for bilirubin production that are slightly lower than those calculated from carbon monoxide production.¹⁷

A major portion of the heme in the body serves as the prosthetic group of haemoglobin and accounts for approximately 70% of the bilirubin excreted in humans. Variable amounts of heme are present in hemoprotein enzymes (e.g. cytochromes) which are essential in aerobic metabolism of all mammalian cells. Apart from the liver, these hemoproteins are so small, or their turnover rates so slow (e.g. myoglobin) that their contribution to the total bilirubin production is minute. Significant amounts of bilirubin are from the degradation of non-haemoglobin heme in the liver. Evidence for this non-erythroid source of bilirubin was first shown by using precursor glycine,^{18,19} radiolabeled and the radioactivity examined in fecal stercobilin. The major peak of labelled stercobilin excretion corresponded to destruction of haemoglobin of senescent erythrocytes (after 100 - 140 days), but between 10 to 20% of the excreted radiolabel in stercobilin appeared within the first few days after injection of the glycine. Production of this "early-labelled" bilirubin (ELB) begins within minutes after injection of the labelled heme precursor, reaches a peak value within one or three hours, and then declines asymptotically over a period of several days.²⁰

The existence of the two or more ELB components has been demonstrated using radiolabelled δ - aminolevulinic acid; this heme precursor is preferentially incorporated into hepatic heme rather than into hemoglobin of erythroblasts.^{21,23} Recent observations are putting the hepatic contribution to total bilirubin formation from 10 to 20%²⁴ to 23 to 37%^{16,25}. Thus the major contribution could be expected from cytochrome P₄₅₀ as these hemoproteins incorporate at least 70% of total heme synthesized in the liver,²⁶ and treatment with xenobiotics that accelerate hepatic cytochrome P₄₅₀ turnover correspondingly alter the shape and magnitude of ELB.^{27,28} In addition, careful analysis of the initial ELB component has revealed that the liver²⁹ and erythroblasts^{30,31} contain a small heme pool that exhibits a turnover rate (60 - 90 mins) exceeding those of any known hemoproteins.^{32,33} It has been speculated that this pool may represent "free" or "unassigned" heme, which is in transit to the subcellular sites of hemoprotein synthesis and has important regulatory functions.³⁴

The erythropoietic contribution to ELB may increase when erythropoiesis is accelerated and/or abnormal (dyserthropoiesis); this is observed in hemolytic disorders such as iron deficiency anemia, megaloblastic anemia, lead intoxication, thalassemia and congenital erythropoietic porphyria.^{35,36} A decreased ELB peak has been found in patients with aplastic anemia.³⁷

Haemoglobin heme is normally degraded to bilirubin in the mononuclear phagocytic cells (reticuloendothelial cells) of the spleen, bone marrow and liver in which senescent erythrocytes are sequestered. Free haemoglobin, on the other hand, released into the circulation by intravascular hemolysis, binds to haptoglobin, and this complex is taken up and degraded predominantly by parenchymal cells.³⁸ Excess haemoglobin may be partially reabsorbed and degraded to bilirubin by the epithelial cells of the proximal convoluted tubule in the kidney.

1: 3 Catabolism of haemoglobin

It has been well-established that heme catabolism in man and other mammals normally results in nearly stoichiometric formation of carbon monoxide and bilirubin,¹⁵ and requires molecular oxygen.³⁹ Furthermore the conversion process is regiospecific⁴⁰ with biliverdin as the intermediate,⁴¹ such that all natural bile pigments have the IX α configuration.

Physiological heme degradation in mammals appears to be catalyzed by two enzyme systems, one microsomal, the other cytosolic. The initial reaction which is an oxidative attack on the α - methene bridge of heme, resulting in cleavage of the ring tetrapyrrole and formation of biliverdin, is catalyzed by heme oxygenase, a microsomal enzyme with the highest activity in the spleen, and other tissues such as the liver, and macrophages.^{42,43} This enzyme is most active on "free" heme or heme loosely bound to protein (e.g. methemalbumin) and fulfills the requirements of converting heme into equimolar amounts of carbon monoxide and bile pigment that has the IX α configuration.

The reaction catalyzed by heme oxygenase seems to be rate-limiting in the overall conversion of heme to bilirubin in vitro; the enzyme activity is inhibited by carbon monoxide and dependent on molecular oxygen and NADPH which is regenerated by NADPH - cytochrome C reductase.⁴³ Although heme oxygenase uses the microsomal electron transport system, the enzyme is distinct from the drug - oxidizing enzyme system of the microsomes and it does not use cytochrome P₄₅₀ as a terminal oxidase.^{44,45} Microsomal heme oxygenase was purified from pig spleen; it has a molecular weight of approximately 32,000 and is free from heme.⁴⁴

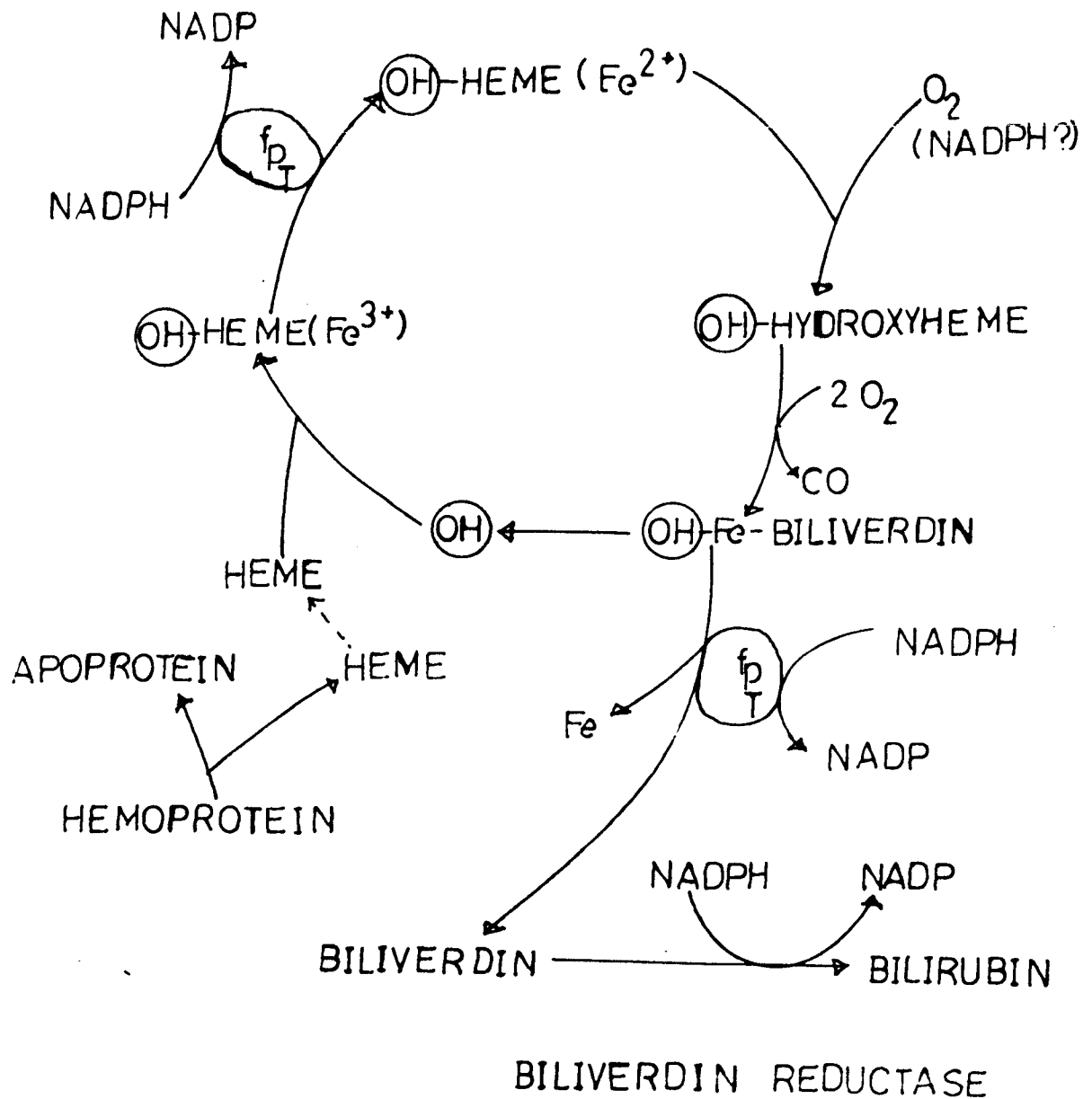
Although the precise metabolic pathway of heme to biliverdin is still unclear, recent animal studies using ¹⁸O₂ and detailed analysis of heme degradationⁱⁿ in vitro model systems has resulted in a substantial progress in the understanding of the pathway. It has been established

that the terminal lactam oxygen atoms of bilirubin are derived from different O_2 molecules,^{39,40} that the initial reaction involves formation of an iron - O_2 complex both in vivo and in vitro,⁴⁶ and that the apoprotein to which heme is attached during its degradation has a profound influence on the regioselectivity of the cleavage reaction.⁴⁷⁻⁴⁹ Current concepts of the heme oxygenase reaction⁴⁵ developed from these studies and from analysis of heme degradation in a reconstituted microsomal heme oxygenase system can be summarized as follows (Figure 3). The heme is postulated to be dissociated from its apoprotein (e.g. globin) and transferred to the microsomal heme oxygenase apoprotein. The microsomal NADPH - dependent system reduces bound (ferric) heme to its ferrous form. The latter form then binds molecular oxygen by coordination to the iron and activation of the oxygen occurs; the mechanism although unclear is thought to involve electron transfer to the heme-oxygen complex in a way similar to that of the cytochrome P_{450} catalyzed hydroxylation. It is also assumed that in the heme-heme oxygenase substrate - enzyme complex heme is attracted to the protein such that the β , γ and δ methene bridges are sterically hindered by the amino acid residues from a bound oxygen molecule.⁴⁸ This arrangement is probably responsible for the preferential attack on the α methene carbon by the activated oxygen species, resulting in formation of hydroxyheme. This intermediate would further react with two molecules of molecular oxygen to produce carbon monoxide and a ferric iron - biliverdin complex. This is followed by the subsequent release of iron, probably in the ferrous form; making this step a rate - determining one in the overall heme oxygenase - catalyzed reaction.

In mammals,^{the} cytosolic enzyme, biliverdin reductase, abundant in the mammalian tissues, further reduces biliverdin to bilirubin in the presence of NADPH.^{50,51} This enzyme displays a remarkable degree of isomer - and

Figure 3

Postulated Reaction Sequence of Heme Degradation To Bile Pigment..



⊙ = HEME OXYGENASE

f_{PT} = NADPH-CYTOCHROME C REDUCTASE

substrate - specificity; unnatural, non - α isomers of biliverdin - IX and biliverdin glucuronides are reduced at much slower rates or not at all.^{52,53} Mesobilirubin, which is formed in vitro by reduction of the bilirubin vinyl groups to ethyl groups, is also derived biogenetically from bilirubin.⁵⁴⁻⁵⁸ The brain tissue was shown to reduce bilirubin to mesobilirubin, in vitro,⁵⁹ which could possibly occur after non - bound unconjugated bilirubin has crossed the blood - brain barrier. Experimental evidence has shown that deeply jaundiced individuals have no bilirubin in their cerebral tissues.⁶⁰

1:4 Metabolism of bilirubin and mesobilirubin

1:4:1. Transport in plasma

From the sites of its formation bilirubin is transported in the plasma tightly bound to albumin and to ligandin in the liver. At physiological pH only minute amounts of unconjugated bilirubin are soluble in protein - free aqueous solutions which have been estimated from titration studies to be 7×10^{-9} M, at 37°C, at pH 7.4⁶¹ and 2×10^{-7} M (0.006 mg%) at pH 7.4, at 25°C ionic strength 0.10M.⁶² The protein, albumin, contains one high-affinity binding site with an estimated affinity constant (K_a) of 1.4×10^8 M⁻¹ in purified human albumin (1.2×10^8 M⁻¹ in albumin of Gunn rat plasma).⁶⁴ Since the concentration of unbound bilirubin is usually very low approximately 10^{-8} M to 10^{-9} M, it is postulated that the cytotoxicity of bilirubin is directly proportional to the unbound bilirubin, thus giving binding proteins an additional function of detoxification. Many endogenous metabolites such as sulfisoxazole⁶⁵ and salicylate⁶⁶ are known to lower the bilirubin binding capacity in a whole serum resulting in a concomitant escape of unbound pigment from the vascular compartment.^{65,66} Long-chain fatty acids are transported bound to albumin and at a concentration range

beyond 4:1 with regard to albumin, they displace bilirubin from the high-affinity binding site.⁶⁷ Thus elevated free fatty acid concentrations in babies receiving parenteral nourishment with fat emulsion or breast-fed infants may result in an increased concentration of unbound bilirubin and development of kernicterus.^{68,69}

Observations suggest that the interaction of bilirubin with albumin is one of the most important determinants for regulating the blood level of bilirubin and consequently for the pattern of pigment distribution among the various body compartments. This has been borne out in studies of unconjugated hyperbilirubinemia in man (Crigler-Najjar syndrome) and rat (Gunn strain).⁷⁰ However, bilirubin in the circulation is normally rapidly cleared by the liver. It is also generally accepted that bilirubin is separated from the carrier albumin prior to its uptake in the liver cell.^{71,72} This hepatic uptake of bilirubin, which is speculated to be carrier-mediated and shared by other organic anions including bromosulphophthalein and indocyanine green,⁷³ greatly exceeds the overall transport maximum; thus making it a non rate-limiting step in hepatic bilirubin excretion.⁷³ The true mechanism of the hepatic uptake is a matter of speculation but there is growing evidence of the existence of bilirubin binding proteins inside the liver. The most abundant one is ligandin.⁷⁴ Ligandin binds a variety of compounds including bilirubin, heme, steroids, bromosulphophthalein and carcinogens. Another protein corresponds to Z protein or fatty acid binding protein (FABP).⁷⁴ This protein, although it binds bilirubin less strongly than ligandin, is principally involved with free fatty acid transport.⁷⁵ The affinity constant for bilirubin of purified rat ligandin (10^6 M^{-1}) is considerably less than that of ligandin in unfractionated rat liver cytosol (10^9 M^{-1}) which suggest that some cytoplasmic property or

constituent(s), perhaps glutathione,⁷⁶ might be required for the preservation of intact binding of bilirubin to ligandin. Rat ligandin is a dimer (molecular weight 46000) composed of two nonidentical subunits that may be responsible for different catalytic and binding functions.⁷⁷ Its physiological role, like albumin in plasma, is solubilization, storage and detoxification of bilirubin; and influencing the net hepatic uptake a concept supported by recent studies using^a multiple indicator dilution technique, which permits individual measurement of influx and efflux.⁷⁸

Physiologic studies on the blood-brain barrier have not provided a precise definition and the concept remains functionally ambiguous. In general therefore, it is useful to consider the blood-brain barrier in relation to the physical-chemical properties of specific types of compounds. The blood-brain barrier plays a central role in the pathogenesis of bilirubin encephalopathy but factors governing the entry of bilirubin into the central nervous system have long been a matter of speculation.⁷⁹ Bilirubin is a highly lipid-soluble, nonpolar molecule which easily crosses biologic membranes, except when bound to albumin. It was shown that the newborn and adult animals did not differ fundamentally in the functional characteristics of the blood-brain barrier for bilirubin. Unbound pigment moves across the barrier rapidly in either direction thus providing strong evidence against the concept of "immaturity" of the blood-brain barrier for bilirubin and therefore, other explanations are required to account for the almost exclusive occurrence of the bilirubin encephalopathy in newborn infants.⁷⁹ However, experimental evidence shows that the kernicteric pigment has the properties of solid mesobilirubin. Mesobilirubin injected intracerebrally in newborn kittens stained the tissue a bright canary yellow.⁸⁰ Very few studies have been done on the association of

mesobilirubin with known carrier proteins, but albumin does bind mesobilirubin⁸¹ and available methods do not differentiate the pigment from bilirubin in serum.⁸²

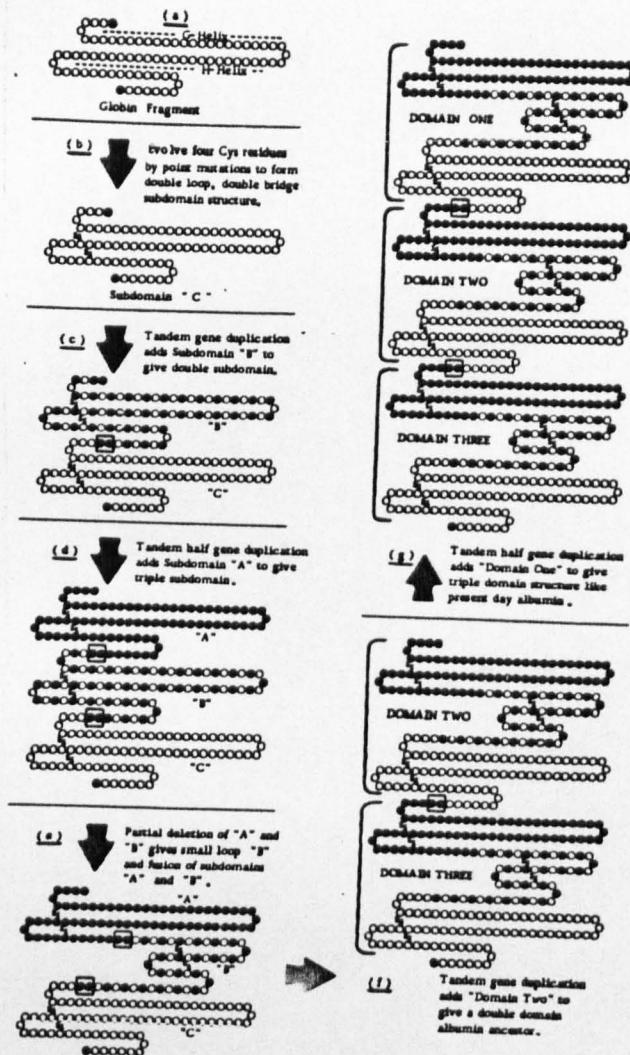
1:4:2. Transport Vehicles

Within the plasma exists the "transport milieu" that is the mobile milieu in which substances and biological units are contained, and which, through transitory bindings, serve the transport processes as such. The vehicles are components of the transport milieu which always have the longer biological half-life. Two transport systems do exist: "the macro-distance transport" - the motive power of which is caused by the pumping of the heart and the "micro distance transport" - which exists within fixed membranes or within the cells themselves.⁸³ Although exceptionally complicated only albumin which is 42 gm/litre in blood serum, is concerned as a binder in the macro-distance transport of bilirubin.

Albumin contains four similar subunits which are covalently joined to make a single polypeptide chain and Peters⁸⁴ recently proposed a four domain model based especially on large fragments which retained structural and functional properties. The amino acid sequence and disulphide bridges of serum albumin reveal a pattern of loops that indicate how the molecule evolved from a pattern of about seventy seven residues by four fold tandem gene duplication and one deletion, to give a protein of about 585 residues (Figure 4).⁸⁴

The sequence and disulphide bridges as shown in Figure 5, reveal a pattern of loops and connecting segments that repeat three times. The three major repeat units, consisting of two large double loops separated by a small double loop, corresponding approximately to residues 1-190, 191-382, and 383-585. One exception to the pattern is the first loop which is missing cysteine residues that should close the large loop at 8 and 54. The bridge was probably lost during evolution.

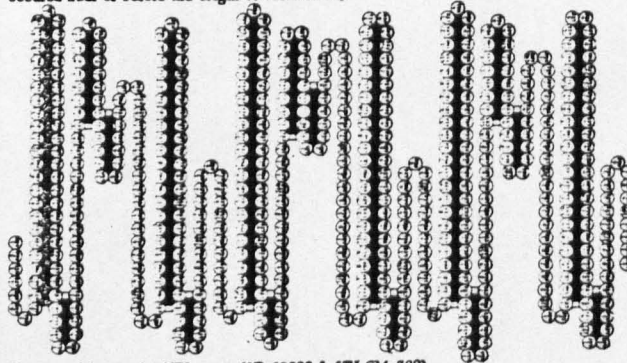
Figure 4



Evolution of serum albumin

Figure 5.

STRUCTURE OF HUMAN SERUM ALBUMIN. P. O. Behren*, A. M. Spiekerman*, and J. R. Brown* (Spons: Lester J. Reed), Clayton Fdn. Bioch. Inst., Austin, Texas 78712. The structure is based on tryptic and chymotryptic peptides. The S-S bridges are based on analogy with BSA (Brown, Fed. Pr. 33:1379, '74). Sequences of some fragments of HSA have been reported (Radshaw & Peters, JBC 244:5582, '69) (Kusnir, et. al. Col. Czech. CC 38: 155, '73; BBA 310:124, '73; FEBS Let. 27:121, '72) (Babin & Goss, Eur. JB 34:409, '73) (Gambhir & McMenamy, Fed. Pr. 32:457, '73); and our sequence is in agreement with all except for several differences with the last reference. The structure below reveals three homologous repeat units or domains (1-191)(192-384)(385-584); and this indicates that gene duplication occurred twice in the evolution of serum albumin. Similarity of the large loops within each domain suggests that these also have a common origin. The large sequence differences between domains (comparable to hemoglobin/myoglobin) suggest that the triplication occurred near or before the origin of vertebrates.



(Supported in part by NIH grants HD-02890 & STI GM-789)

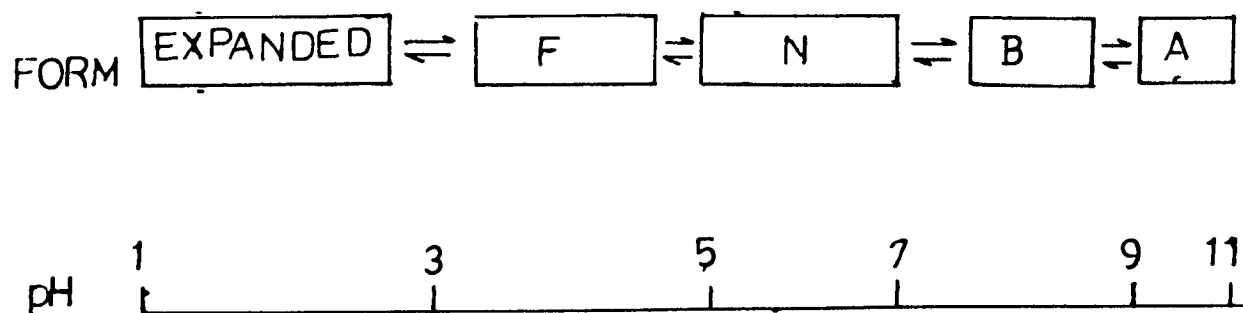
Amino Acid Sequence and disulphide bridges of Human serum albumin

Thus the protein may be considered to contain nine domains corresponding to the six large and three double loops.⁸⁵ This repetitive nature of the albumin structure, the high helical content of 54-68%, and the restrictions imposed by the double cysteine bridges greatly simplify consideration of three dimensional models.

Crude commercial preparations of albumin are heterogenous due to impurities such as α -globulins, enzymes, dimers, mercaptalbumin and in some cases abnormal albumins. This heterogeneity can be avoided by using crystalline or chromatographically purified albumin. The most studied cause of heterogeneity is isomerisation (Figure 6). Essentially this isomerisation exists from the simple, reversible expansion to irreversible disulphide rearrangement. At pH 5-7, the normal, N form predominates and below pH 4 appears the F, for fast-migrating form which is somewhat expanded; below pH 3 the molecule expands further to expose most tyrosines and other hydrophobic residues to the solvent. At higher pH values, near pH 8, particularly in the presence of calcium ions, a different expansion, with increased accessibility of hydrogen atoms for exchange, increased mobility of the thiol group and the slight loss of helix, produces the B form. The B form, is however, readily reversible. Above pH 8 a slow transformation to the A form occurs. This A form is the opposite of the F form, described above, that is, slightly slower migrating than the N form on electrophoresis (pI of 5.45 compared to 5.24).⁸⁶ Disulphide bond rearrangement is involved since the A transformation is accelerated by small amounts of thiol compounds and suppressed by alkylating agents such as iodoacetamide. The change to the A form becomes irreversible near pH 10. However, the A form disappears faster than the normal or N form in vivo and suggests that it may be an intermediate in albumin degradation.⁸⁷

The present knowledge of the structures of human and bovine albumins

Figure 6



Isomeric forms of albumin observed at different pH values.

is accredited to Brown and his colleagues⁸⁸ and Meloun's group.⁸⁹ Both teams noted the regular occurrence of adjacent Cys-Cys sequences which are the basis of the repeating loop structure. The loops are numbered sequentially from 1 to 9 rather than by domains; thus, loops 1 -3, 4 - 6 and 7 - 9 are domains I, II, and III respectively (Figure 5).

The secondary structure of albumin was estimated by circular dichroism (CD), predictions from the amino acid sequence and model building. The total of 50 - 55% α - helix and 15 - 18% β - sheet derived from optical measurements, approximates the predicted total from amino acid sequence of 46% α and 16% β for bovine albumin.

The stability and flexibility of albumin in the face of conditions which would denature many other proteins is attributed to the loop structure. An isolated fragment loops 7 - 9 of the bovine protein shows the strong primary site for palmitate and an amino terminal fragment. Loops 1 -5, binds bilirubin as strongly as does the native albumin.⁹⁰ Similarly, at least six antigenic sites appear to be preserved in fragments some as small as one loop in size.^{91,92} The autonomy of local regions is not dependent solely on S - S bridging, since even isolated fragments can refold to the native disulphide pattern after complete reduction of disulphides; as attested by the return of antigenic sites⁹¹ and of native circular dichroic pattern and fatty acid binding.⁹² The return to the native structure is however slower in the albumin than in the fragments after reduction. Hence the refolding probably initiates in the local regions, so that the individual loops form their S - S bonds rapidly and semi-independently, preventing the occurrence of unnatural S -S bond between loops or the intertwining of strands through loop openings. That the helix appears before palmitate binding ability returns is in agreement with the concept that secondary structure forms before tertiary structure.⁹²

Expansion at acid or alkaline pH, for example, is probably a repulsion of loops as salt bridges are broken. Formation of the A isomer above pH 8 is believed to involve the single thiol at Cys₃₄ as a catalyst to effect an interchange between two disulphide bonds depicted in Figure 7.

Figure 7.

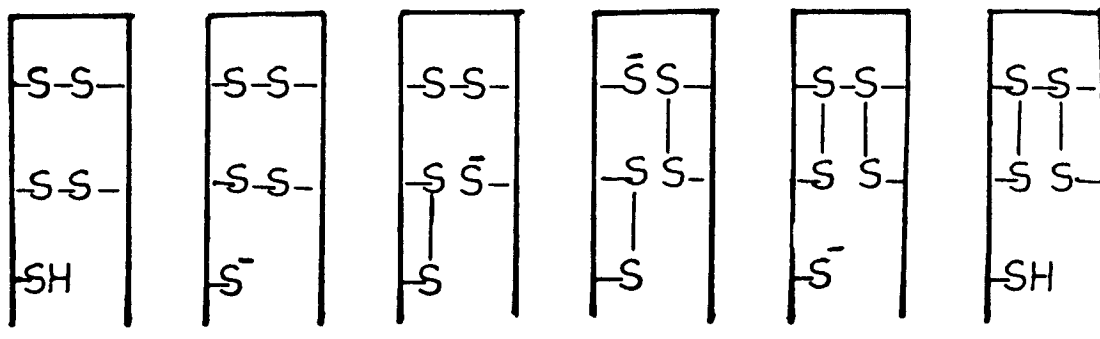


Diagram of a possible base-catalysed disulphide interchange model, showing how the thiol group may effect the interchange of two S-S bonds yet remain itself unchanged.

Taken from Ref. (92)

This interchange has been shown to occur only in the first domain. The N - terminal position of the albumin molecule is relatively compact compared to the C - terminal region. Further evidence suggests that the C - terminal portion unfolds more extensively than does the N - terminal portion during the N - F transformation.⁹³ Much of the compactness of the N - terminal is due to the hydrophobicity and the numerous aromatic residues of loop 3. The tryptophan in loop 3 (bovine albumin) the analogous residue is absent in human albumin, fluoresces more strongly than the one in loop 4.⁹⁴

On the basis of the current information the locations of sites on the albumin molecule are divided into the following categories:

a) the hydrophobic, non-covalent sites for (1) primary long chain fatty

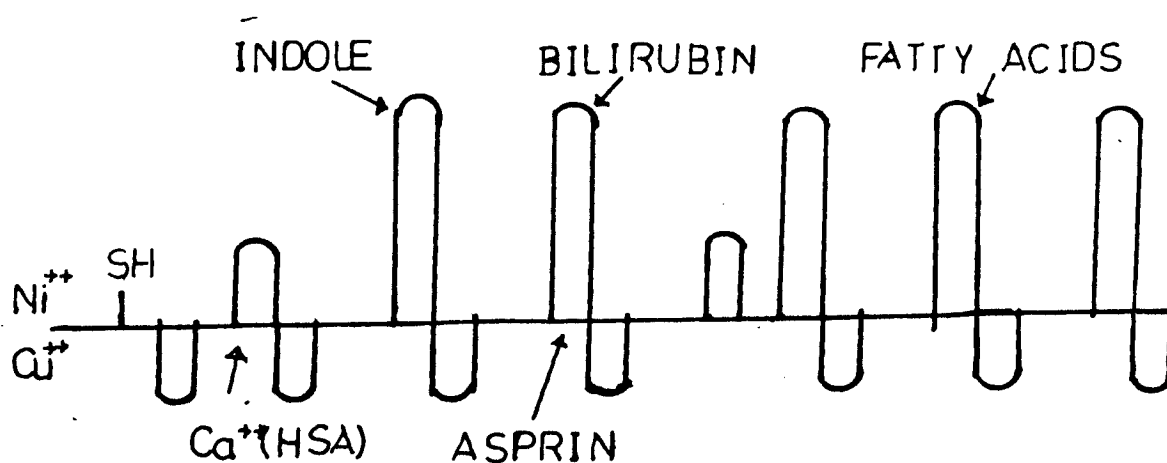
acids, (2) bilirubin and certain drugs, (3) indole compounds and certain other drugs;

(b) covalent attachment sites for organic ligands including the thiol group;

(c) chelation sites for divalent metals, see Figure 8.

(d) sites for organic dyes. The binding of the dyes is useful for physico-chemical, medical or analytical studies. The association constants decrease from 1.7×10^7 to $2.8 \times 10^4 \text{ M}^{-1}$ for the compounds sulphobromophthalein (BSP)⁹⁵, bromophenol blue⁹⁶, Evans blue⁹⁷, bromocresol green⁹⁸, methyl orange⁹⁹ and phenol red.¹⁰⁰ Thus the specificity for certain dyes is used for albumin assays in the plasma.

Figure 8



Model of principal ligand binding sites of albumin.

The assay of albumin bromocresol green gives linear results and is free from interferences,^{101,102} unlike the separation from globulins and subsequent measurement by general methods of proteins.

1:4:3. In vitro and In vivo Bilirubin-Albumin Binding Studies:

A number of methods have been used to measure the binding of bilirubin to albumin, such as quenching protein fluorescence¹⁰⁸, circular

dichroism,¹⁰⁴ ultrafiltration,¹⁰⁵ ultracentrifugation,¹⁰⁶ dialysis,¹⁰⁷ electrophoresis,¹⁰⁷ gel filtration,¹⁰⁸ electron absorption spectrophotometry,^{103,109} spectrofluorimetry,^{103,109} spectropolarimetry,¹¹⁰ competitive binding to calcium carbonate¹¹¹ and affinity chromatography on agarose - albumin polymers.¹¹²

This binding is reversible and it is accompanied by development of a characteristic absorption spectrum ($\lambda_{\text{max}} \simeq 460 \text{ nm}$ at pH 7.4),¹⁰³ appearance of optical activity in the visible light region,¹⁰³ enhanced circular dichroism,¹⁰⁴ development of bilirubin fluorescence,¹⁰³ and protection of the pigment from photochemical autoxidative, and enzymatic degradation.¹¹³

There is little agreement on the binding capacity of serum albumin for bilirubin and the size of the corresponding association constants. Despite all the work done in the binding of bilirubin to albumin, investigations have tended to be obfuscated and bedeviled by the physical and chemical instability of bilirubin in aqueous solutions and its tendency to adhere to many surfaces, including glass. This, therefore, calls for critical evaluation of the techniques used in the binding studies before conclusions can be drawn. Much of the disagreement lies around the role of crude and impure albumin and bilirubin preparations; added to this is the difficulty in measuring unbound pigment at low bilirubin-albumin ratios. There is, however, a general agreement that pure albumin binds at least one molecule of bilirubin tightly.

Most of the methods used for in vitro binding studies of bilirubin to albumin have certain anomalies and of the spectroscopic methods that have been used the fluorimetric technique¹⁰³ has the greatest appeal in terms of simplicity, sensitivity, and reliability. The only chemical method that has been applied is the peroxidase method.^{64,114} This technique evolved from the observation that bilirubin is oxidized by

hydrogen peroxide or ethyl hydroperoxide in the presence of peroxidase much faster when albumin is absent than when it is present.^{113,114} It is based on the assumptions that the initial velocity of the enzymatic oxidation in the presence of albumin is slower than the dissociation of bilirubin from albumin (i.e. rate limiting) and directly proportional to the concentration of unbound bilirubin.⁶⁴ Since the method requires measuring the rate of oxidation of bilirubin in the presence and absence of albumin, difficulties may be experienced at physiological pH because of the instability of bilirubin in the albumin-free solutions.¹¹⁴ Association constants obtained by the peroxidase method tend to be a magnitude higher than those obtained by other methods.

For purified human albumin the association constant is in the range $10^7 - 10^8 \text{ M}^{-1}$. This indicates that the concentration of unbound bilirubin in a solution containing bilirubin and albumin at a ratio of 1:1 will be less than $10^{-6} - 10^{-7} \text{ M}$. For a bilirubin-albumin ratio of 1:2, the unbound bilirubin concentration will not exceed $10^{-7} - 10^{-8} \text{ M}$, and for a solution containing 0.60 mM (3.99g%) albumin and 0.17mM (10mg%) bilirubin (typical values for rather jaundiced serum) the "free" bilirubin would be approximately $4 \times 10^{-8} - 4 \times 10^{-9} \text{ M}$.¹¹⁵ The variation with temperature of bilirubin binding affinity to human serum albumin seems to indicate that the binding forces are enthalpic and, therefore, predominantly of an electrostatic nature.

Only purified human adult albumin will be dealt with in this thesis.

Binding studies in vivo, i.e. in the presence of serum have been done using a more rapid micromethod for measuring the reserve albumin binding capacity with the dye, 2 - (4 - hydroxybenzene azo) benzoic acid (HBABA). This method has been used in infants who developed kernicterus and were shown to possess no or low binding capacity.¹¹⁶ A more common method for the determination of unconjugated serum bilirubin has been carried out

using chromatography on Sephadex G-25. Data obtained by this method indicates that the amount of bilirubin, both in vivo and in vitro, varies directly with the level of total bilirubin.

Using a dialysis technique purified fetal human albumin prepared from umbilical cord blood was shown to have a greater affinity for bilirubin than adult human albumin.¹¹⁷ The affinity of albumin for bilirubin is dependent on the medium, and the effective binding capacity of albumin for bilirubin can be reduced by substances that compete with bilirubin for binding. The binding capacities of purified human adult albumin in buffer and the albumin in adult human serum were shown by circular dichroism, peroxidase and chromatography on Sephadex G-25 to be identical.^{114,118,119} However, measurements with an indirect fluorimetric method do not support the above conclusion, but instead indicate that the capacity of the albumin in adult serum is about twice the capacity of the purified protein.¹²⁰ The following tentative conclusions can be drawn from studies published so far: (a) the presence of normal serum has little effect on the binding capacity or affinity of albumin for bilirubin, except perhaps to increase the latter.

(b) Unidentified factors may occur in neonatal serum that decrease the effective capacity of the first high affinity binding site.

The binding affinity of albumin for bilirubin is decreased by the presence of dimethylsulphoxide,¹¹⁷ and varies with ionic strength.¹¹⁸ Increase in temperature tends to decrease the affinity. Although circular dichroism and fluorescence spectra of bilirubin-albumin complexes are pH dependent^{104,109,118} the binding characteristics of the primary binding site of serum albumin appear to be independent of pH over the range pH 7 - 9.^{119,128} Nevertheless, acidosis in vivo favours a shift from serum albumin to fat.

In human serum albumin the high-affinity site seems to be a hydrophobic crevice located about 28 Å⁰ from the single tryptophan residue,¹⁰³ and with arginine, tyrosine, histidine, and lysine residues in the more immediate vicinity.¹²²⁻¹²⁴ Little else is known about the geography of the binding site except that its chirality at physiological pH is not the same for albumins from different animals.^{103,118} Bilirubin conjugates¹²⁵ and bilirubin dimethyl ester¹²⁵ bind to albumin and bilirubin covalently anchored to a polymer support via its propionic acid side chains binds albumin.^{112, 126} Quantitative studies have shown that mesobilirubin also binds to albumin.⁸¹ Hydrophobic interactions,¹¹¹ and perhaps hydrogen bonding between the lactam groups of bilirubin and amine groups of the protein¹²⁷ seem to be more important. The change from the methene to methylene bridge (position 10, Figure 1 structure 3) in the tetrapyrrole pigment gives biliverdin and the latter forms a non-ultrafiltrable complex with albumin.¹²⁸ There is also strong evidence that the carboxyl groups of bilirubin, in the serum albumin-bilirubin complex, are oriented towards the aqueous phase of the medium. Although the conformation of the bound pigment (bilirubin) is not known, it may be inappropriate and misleading to consider bilirubin as a typical "organic anion" with respect to its complexation to albumin, at least at the high affinity site. Recent studies, however, point out that bilirubin acid releases two protons on binding to albumin¹²⁹ which is stoichiometrically equivalent to binding bilirubin dianion.

The binding of bilirubin has a marked effect on its chemical reactivity. Whilst it is known that bilirubin is chemically unstable in water, the pigment is relatively stable in chloroform or when it is bound to albumin. The reason for this stabilising effect due to the protein is not known. Probably the pigment assumes a stable, introverted, intramolecularly

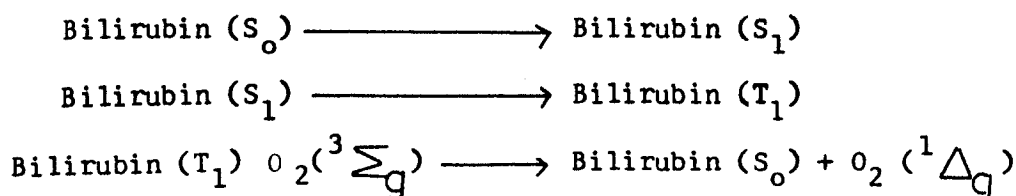
hydrogen-bonded structure when bound, or perhaps its sensitive and vulnerable parts, especially the central bridge, are protected by the lipophilic environment of the albumin interior. This lipophilic environment of the albumin interior binding site is known to be on or around lysine - 240 of the albumin molecule.¹²⁴

1.5 Photochemistry of Bilirubin

The realisation of yellow jaundice was first by Galen and dates back as far as Hippocratic medicine (460 - 370 B.C.). Thus for centuries the view of jaundice continued and it was not until 1807 when Thenard studied the yellow pigment of bile isolated from bovine biliary calculi.¹³⁰ It was in the hope of treating neonatal jaundice by phototherapy that stimulated a sudden and continued interest of the photochemistry of 5, 15 - biladienes, bilirubin IX α in particular.

Bilirubin in water or common organic solvents at room temperature does not fluoresce.¹⁰³ On the other hand, solutions of bilirubin in which the pigment is solubilized with detergents¹³¹ or by binding to serum albumin,^{103,118} and basic solutions of bilirubin in organic solvents, (e.g., 1% NH_4OH - MeOH, 10% triethylamine-chloroform) do show a distinct fluorescence that is clearly visible and is most intense at about 510-530.^{118,131} Clinically, this has been of great importance for the determination of the reserve binding capacity of newborn serums.

In the presence of oxygen, bilirubin has been known to photosensitize the formation of singlet oxygen.^{122,134} The following sequence of reactions is thought to occur:

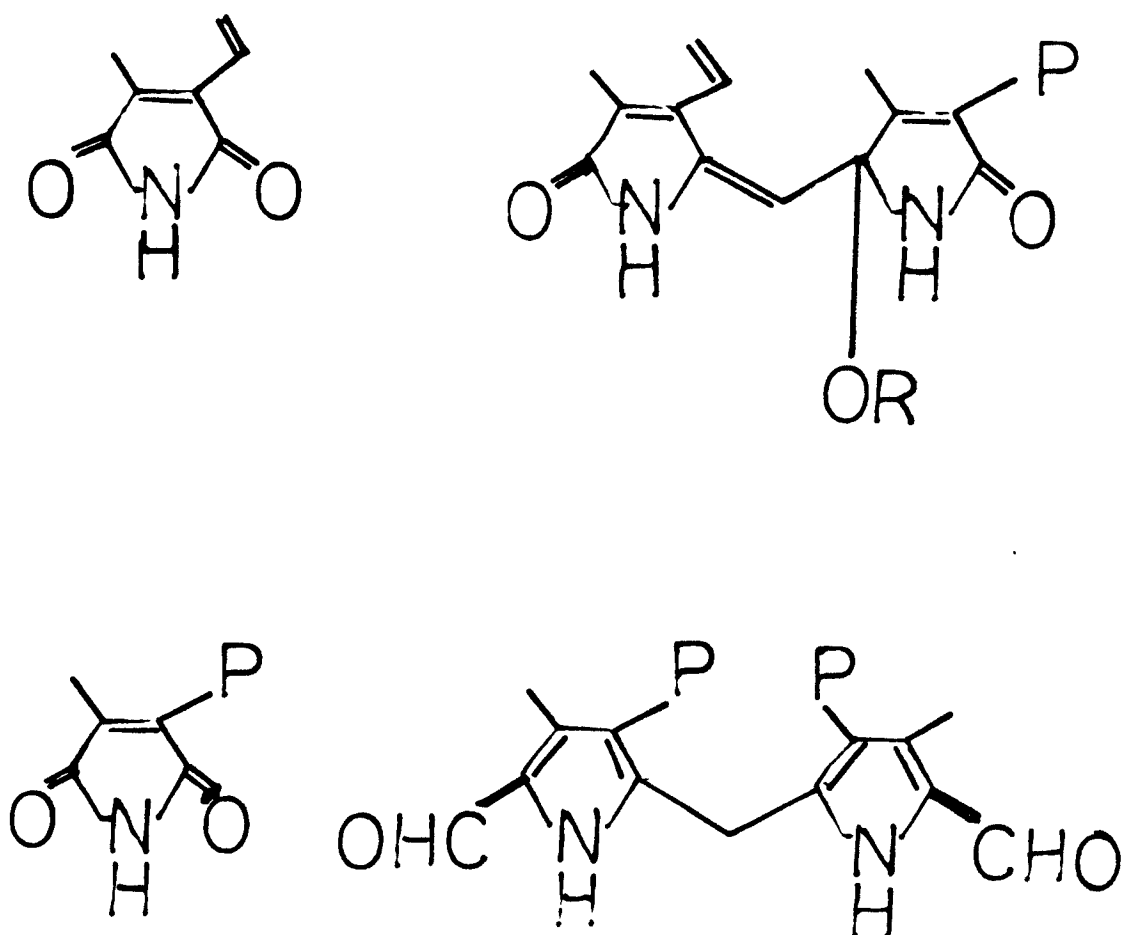


1:5:1. Photooxidation

One of the major problems met by scientists working with bilirubin is its photooxidation and photodegradation on exposure to light. The rate of decomposition however, depends on several factors: the physical state of the pigment, its concentration, its environment, the oxygen concentration, and the wavelength and intensity of the ambient light. Photodegradation occurs slowly in the presence of albumin and much faster in dilute solutions in organic solvents and this is under both aerobic and anaerobic conditions.¹³⁵ So far photooxidation of bilirubin produces predominately biliverdin as the product although this reaction depends on the conditions employed. High concentrations of the starting material and nonpolar solvents appear to favour biliverdin formation, and, since the reaction is probably a free-radical process it also should be favoured by binding of bilirubin to albumin. It has however not been established whether oxygen is required for the photooxidation of bilirubin to biliverdin. The photooxidation of bilirubin often competes with its photooxygenation and the major products in the latter reaction have been shown to be the mono- and dipyrrolic structures (Figure 9)^{136,137}

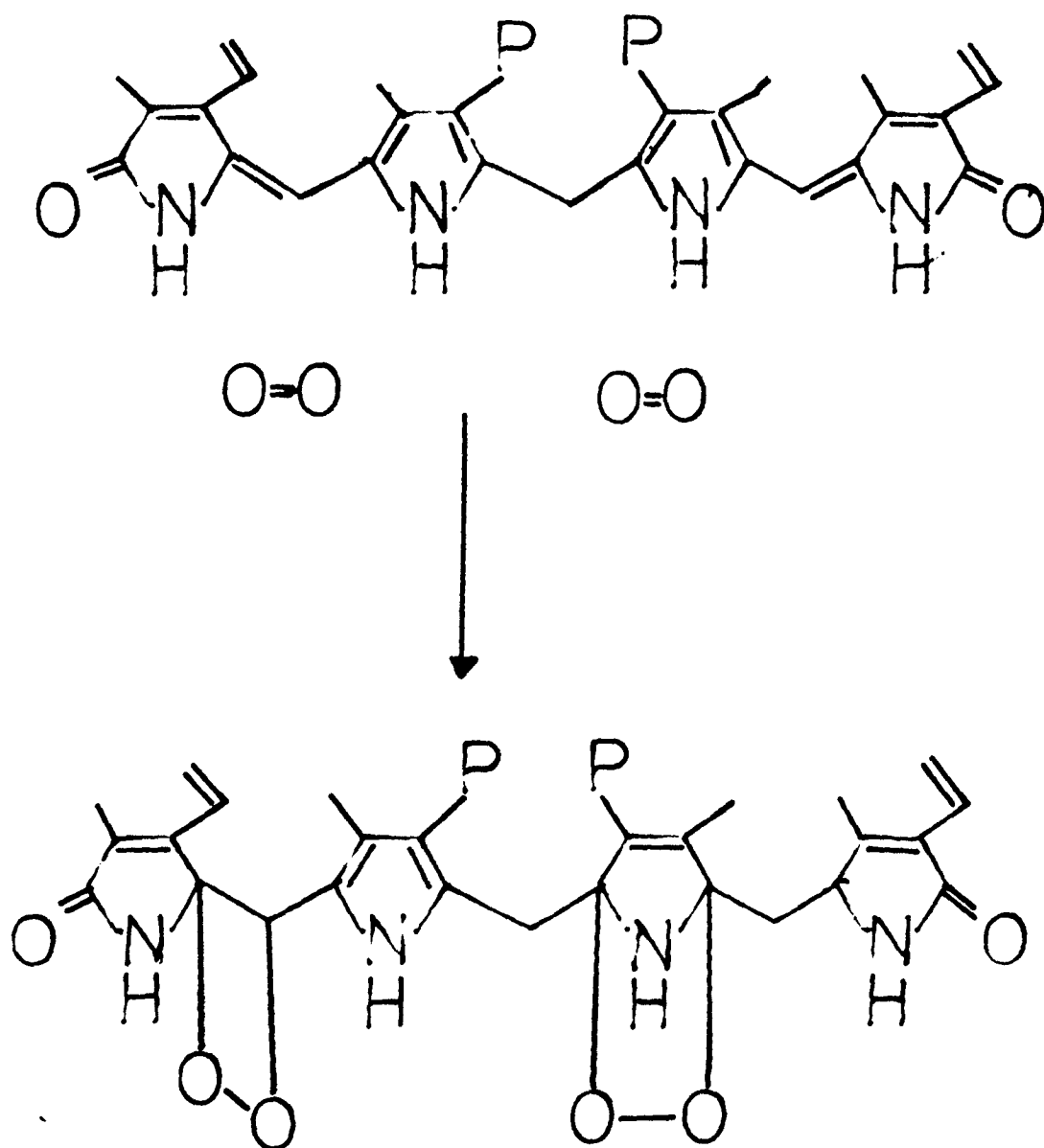
The predominant modes of attack of singlet oxygen on bilirubin are evidently 1, 4 - addition across the pyrrole rings and 1, 2 - addition to the bridging double bonds as indicated on (Figure 10). The intermediate adducts are probably transformed into final products by thermal reactions. The intermediate adducts have been isolated from the photooxidation of bilirubin - albumin solutions,¹³⁸ and the partially characterized pigments isolated in low yield by Berry et al¹³⁹ from an irradiated solution of bilirubin in chloroform may be related to these obligatory dioxetan intermediates. The mechanism of the photooxidation of protein - bound or complexed bilirubin has not been established.

FIGURE 9



Products formed on photooxygenation of Bilirubin IX α in hydroxylic solvents; R H or CH₃ depending on the solvent. Only one propentdyone adduct isomer is shown (top right), although other positional isomers are formed.

FIGURE 10



Predominant models of addition of singlet oxygen to
bilirubin IX α .

1:5:2. Photoproduct formation

Bilirubin in chloroform absorbs at approximately 450 nm but when solutions of it in buffer (pH 7.4 or 8.5) aqueous albumin or rat serum are irradiated for six hours with a 400 - watt high pressure mercury lamp with UV filter, the pigment is partially converted to a compound with an absorption maximum (in chloroform) at 442-444nm. The yellow pigment is probably identical with the unidentified compound which was isolated by ultrafiltration from irradiated solutions of bilirubin in aqueous albumin.¹⁴⁰ This photoproduct has the same properties as the natural pigment, that is it dissolves in chloroform and can be chromatographed on polyamide layers. However, unlike the natural pigment, it is unstable on silica gel layers, it is extractable from chloroform solutions into 0.1M aqueous sodium hydrogen carbonate, and fails to give diazo and pentdyopont tests. This same pigment has not been detected in the serum of normal or bile-duct ligated Gunn rats following prolonged exposure of the animals to illumination.¹⁴¹

1:5:3. Photoaddition and Photoisomerisation

Exposure of bilirubin IX α to illumination in the presence of alcohols, or thiols leads to a stereospecific addition to the exo-vinyl group (Figure 11).¹⁴²

This reaction is thought to proceed via a prototropically rearranged intermediate (II) and to constitute a rare example of photochemically induced ionic addition to a vinyl group. Methanol and 2-mercaptoethanol are representatives of the two classes of adducts that have been used, for these reactions which have to be carried out under inert atmosphere to avoid the more facile photooxidation of the starting material and products.

Photoaddition to the endo-vinyl group of a rubin also in chloroform

FIGURE 11

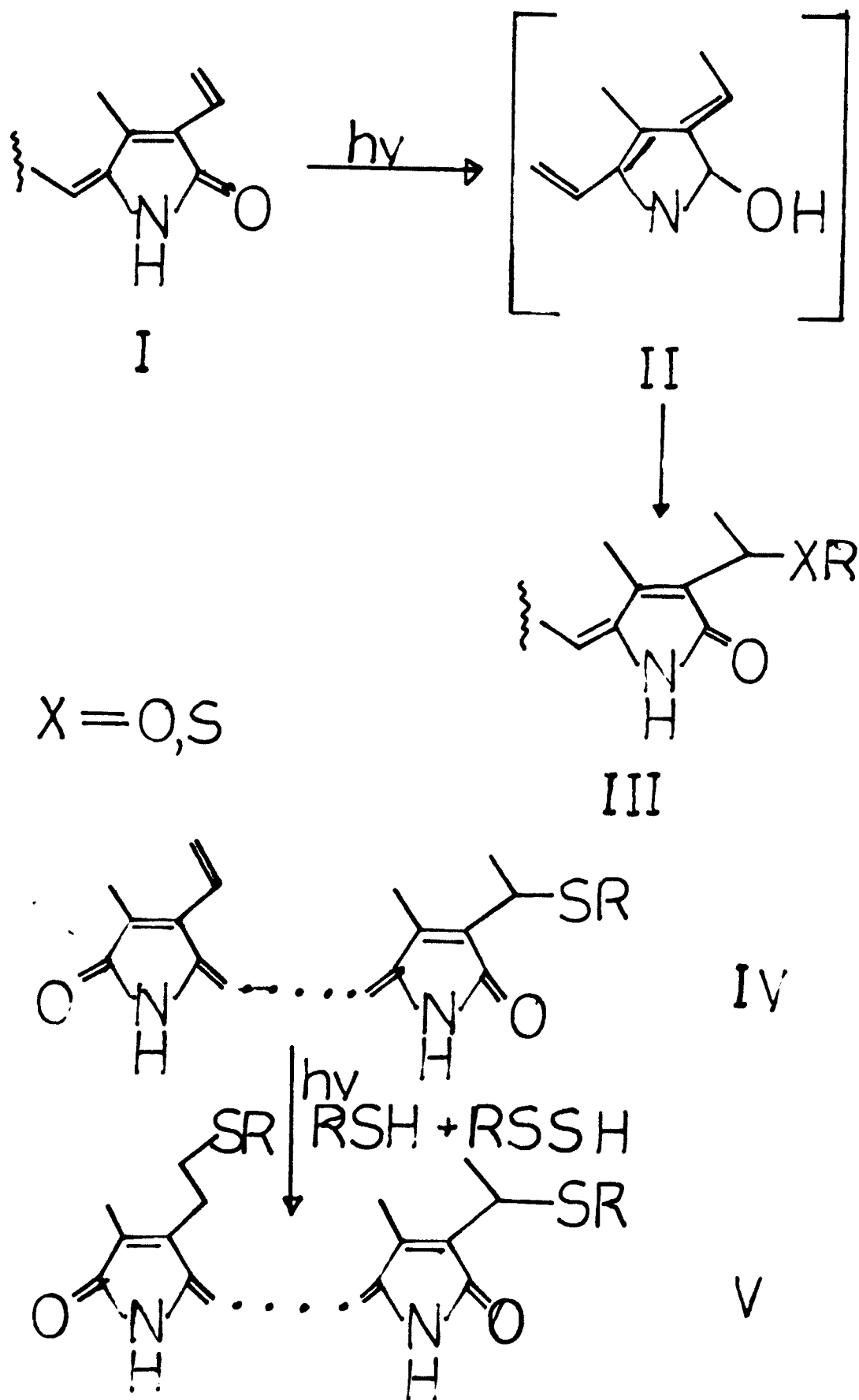
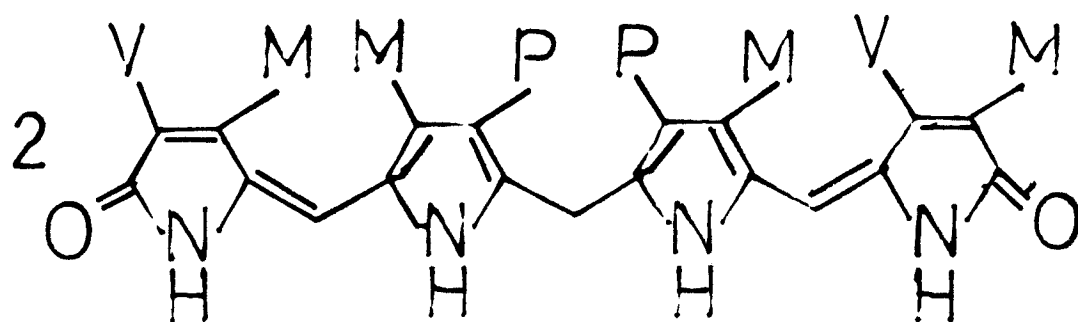
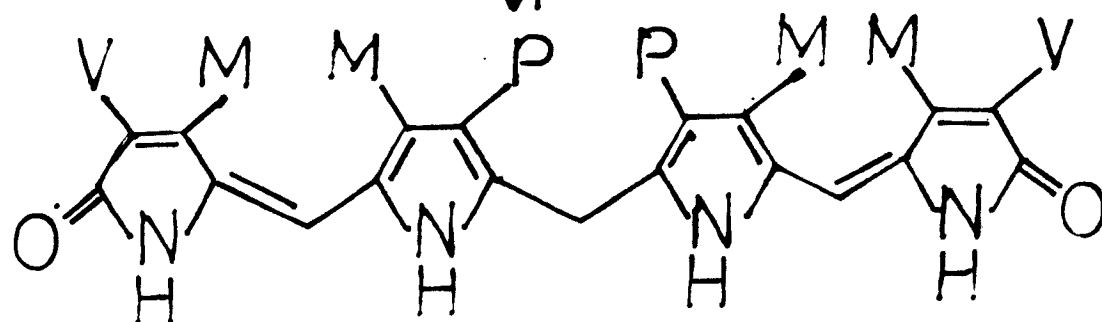


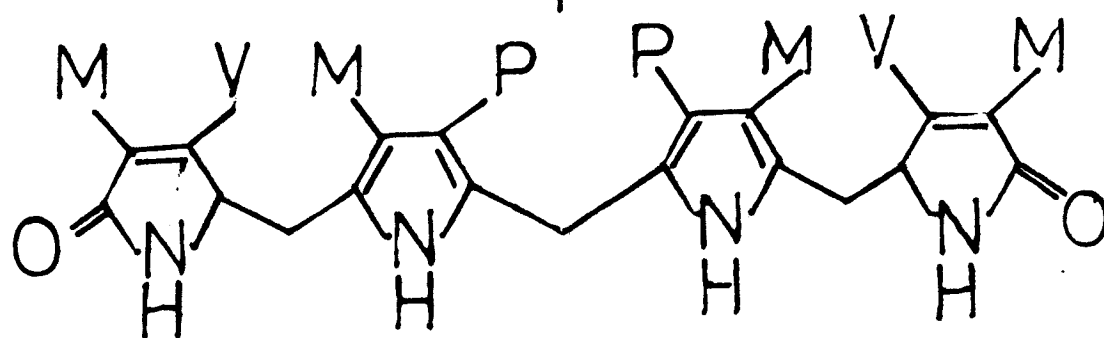
FIGURE 12



BILIRUBIN IX_α



BILIRUBIN III_α



M=CH₃ V=CH=CH₂ P=CH₂CH₂COH

BILIRUBIN XIII_α

Interconversion of bilirubin isomers by Disproportionation.

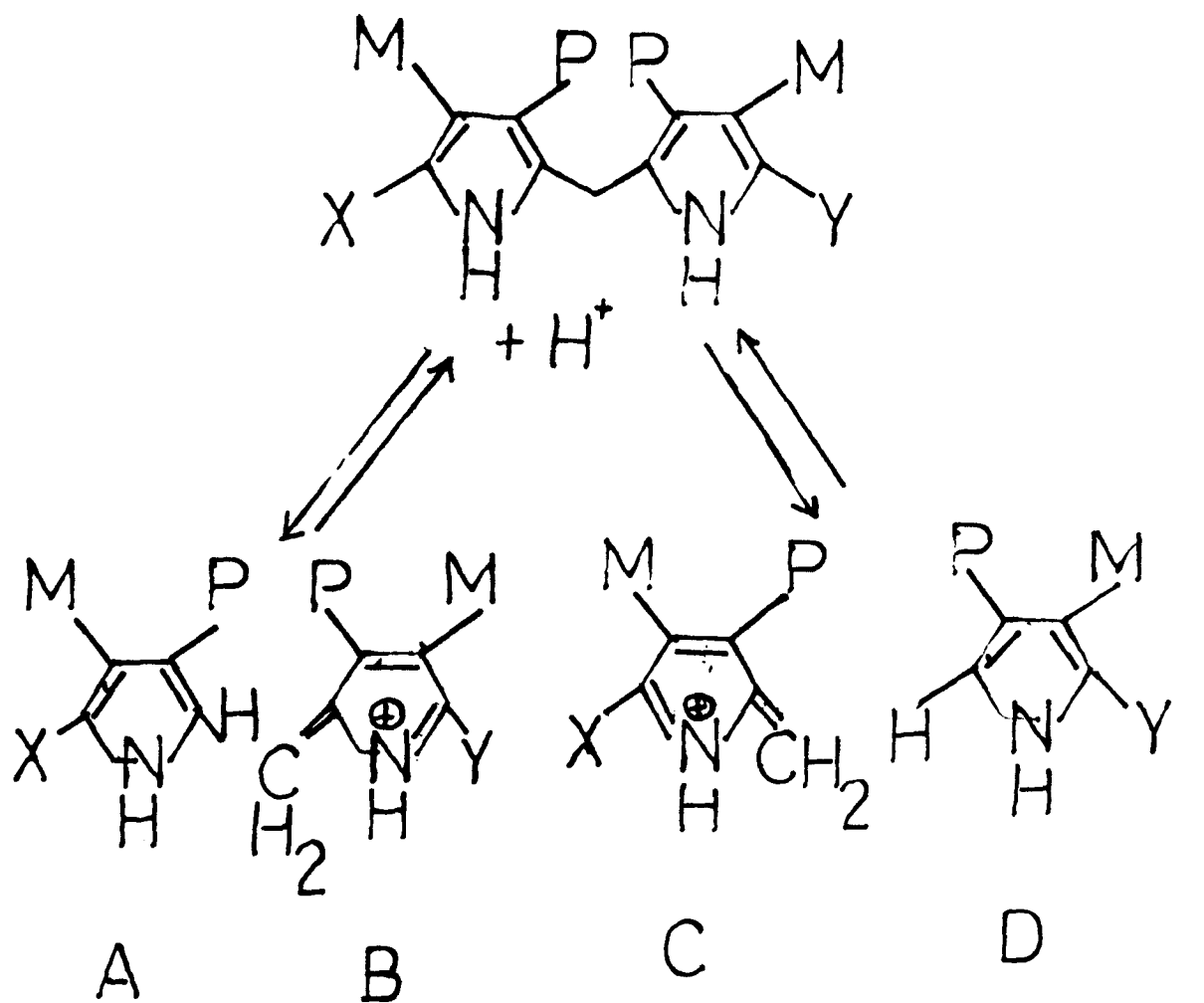
has been observed using a large excess of thiol in the presence of a disulphide and a rubin without an exo-vinyl substituent. (IV \rightarrow V, Figure 11).

Irradiation of bilirubin IX α in deoxygenated aqueous buffer (pH 7.4 - 8.5) gives the isomers bilirubin III α and bilirubin XIII α . This is a reversible reaction which is a counterpart of the oxygen-initiated free-radical disproportionation. The same reaction occurs in chloroform and chloroform - methanol although more slowly.¹⁴³ In sodium hydroxide, (0.1M) or dimethylsulphoxide, anionic and neutral detergents, or if the bilirubin is bound to serum albumin in aqueous solution, the reaction does not take place or occurs at an extremely slow rate. However, addition of acid to bilirubin IX α leads to a mixture containing all the isomers.^{144,145} Thus mixtures of symmetrically substituted rubins can be converted to mixtures of unsymmetrical compounds (Figure 12).

The mechanism resulting from acid-catalysed isomerisation, depending on which side of the C-10 bridge electrophilic attack takes place will give the dipyrrolic fragments as shown in (Figure 12). In bilirubin IX α the rate of proton attack at C-9 and C-11 are similar because these positions are virtually identical and cleavage of the molecule occurs randomly at either side of the bridge. Mixing and recombination of the fragments is also more or less random in concentrated solutions (5 mg/mL) at room temperature. Hence the product contains a distribution of isomers that is almost statistically random.

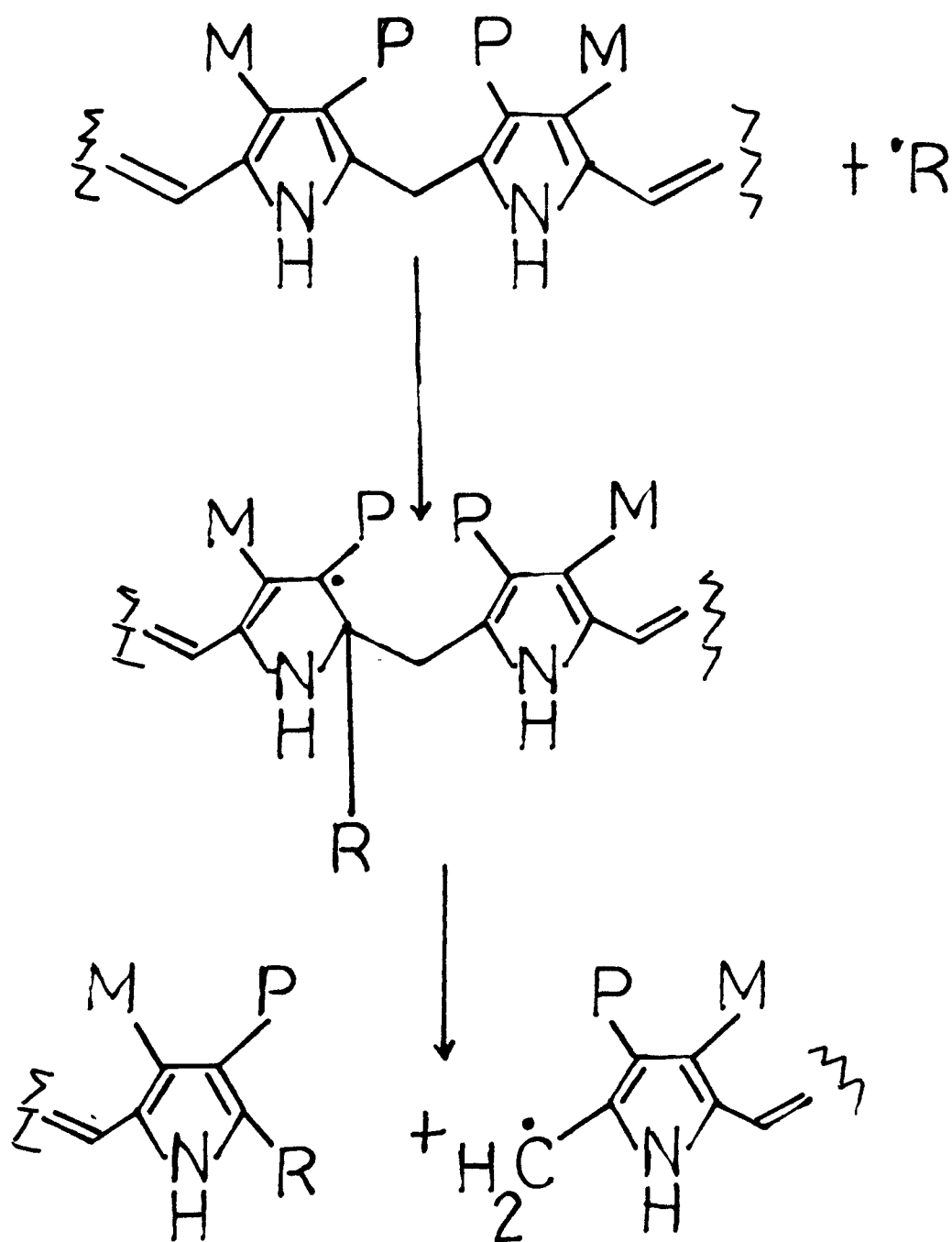
Treatment of bilirubin IX α with hydrochloric acid for one minute gave a mixture (87% yield) containing 23% bilirubin III α , 49% bilirubin IX α , and 29% bilirubin XIII α , which is close to the theoretical distribution of 1:2:1 for a completely random cleavage and recombination process.¹¹¹ The slight preponderance of XIII α over III α is perhaps due to the destabilizing effect of the vinyl groups in the latter. The recovery of

FIGURE 13



Acid-catalysed isomerisation of 5,15-biladienes.

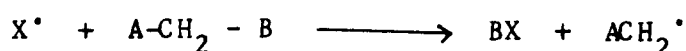
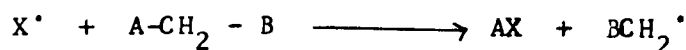
FIGURE 14



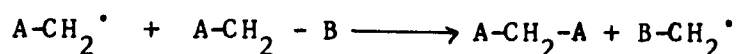
Possible mechanism of the propagation (R^\bullet) step
in the free-radical isomerisation of bilirubin IX α .

bilirubin is inversely proportional to the time of treatment with acid and so does the deviation from the theoretical distribution resulting in an increased proportion of bilirubin XIII α . The rate of acid-catalysed isomerisation decreases with the dilution of the pigment and is low with weaker acids such as acetic acid.¹⁴⁴ Bilirubin IX α treated with p-toluenesulphonic acid in chloroform does not isomerise.¹⁴⁵

Free - radical isomerisation which is different from acid-catalysed process in that it is slower, occurs in water or boiling pyridine in the dark and does not take place if oxygen is rigorously excluded from the solution. Thus the overall isomerisation reaction depicted on (Figure 14) takes place when bilirubin IX α is warmed in moderately basic aqueous solutions (pH 7.4-12). This reaction which is thought to be initiated by oxygen,¹⁴⁶ is inhibited by ascorbic acid, glutathione and thiourea and can be promoted even in the absence of oxygen by benzoyl peroxide, iodine, nitric acid or light. Although the mechanism is unclear, one suggestion is that initiation leads to formation of resonance stabilized dipyrromethene radicals,



which can then react with other pigment molecules,



by a bimolecular substitution reaction (Figure 12) to give isomers.¹⁴⁷

The free-radical isomerisation of bilirubin is dependent on concentration, pH, and solvent. In very dilute solutions (2.1 μ M) in water, the reaction is very slow and loss of pigment and autoxidation predominates. The reaction does not occur in neutral organic solvents such as chloroform and ceases when the pigment is bound to albumin in aqueous solutions.¹⁴⁶

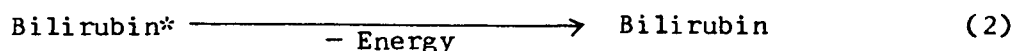
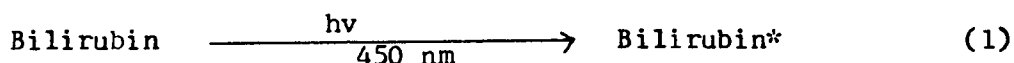
It is this reaction which is likely responsible for the occurrence of bilirubin III α and XIII α in commercial preparations.

Because of its low solubility in water (7nM) bilirubin has been solubilized in water by surfactants (e.g. sodium deoxycholate) giving clear micellar concentrations.¹⁴⁸ Complete solubilization occurs at surfactant concentrations greater than the critical micelle concentration, i.e. 72.4 mM for sodium deoxycholate and anionic (bile acid salts, sodium dodecyl sulphate), neutral (Tween 20, Triton X - 100) and cationic (cetyltrimethylammonium bromide, cetylpyridinium chloride) detergents can be used. There is also an increasing interest in the use of micellar systems to simulate some of the electrostatic and hydrophobic environments found in biological systems in order to elucidate their effects on the kinetics and mechanism of photoreactions.¹⁴⁹ Thus, it has been demonstrated that singlet oxygen, produced in an aqueous solution, can penetrate into the interior of anionic or cationic micelles and oxidize substrates dissolved therein;^{150,151} singlet oxygen can also be generated inside a micelle and diffuse through the aqueous medium to a different micelle. Irradiation of bilirubin or hematoporphyrin in the presence or absence of detergents causes the photooxidation of the pigments.¹⁵² In the absence of dispersed micelles the photooxidation is mainly that involving electron transfer from the triplet pigment to the substrate (radical-involving). The singlet oxygen (1O_2) - involving type was of minor importance.¹⁵² Thus depending on the environment and substrate the two mechanisms may operate together or independent of each other, although there is growing evidence that the radical - involving pathway superceeds the 1O_2 - involving pathway in tetrapyrroles dissolved in detergents, at pH 10. In some further attempts to simulate physiological conditions for phototherapy, certain investigators used erythrocytes which are known to bind significant amounts of bilirubin.¹⁵³ These cells

used in vitro, would serve as models for testing the bilirubin possible phototoxic effects. Thus bilirubin caused the lysis of the haemoglobin-free erythrocyte ghosts as shown by the leakage of enzymes, with concomitant cross-linking of proteins.¹⁵⁴

The photooxidative enamine cleavage was also demonstrated with mesobilirubin from which was isolated methylethymaleimide.¹⁵⁵ This circumvented the vinyl reactivity as mesobilirubin has ethyl, rather than vinyl groups.

Obviously, what goes on during jaundice phototherapy is still a matter of speculation. In vivo studies are compounded by the fact that one or all processes, depending on the environment, may occur, Equations 2 - 4.



Radiationless decay back to ground state, Equation (2). This probably leads to the currently observed geometrical isomers.

Photoaddition, Equation (3). Photooxidation, Equation (4).

(Asterisk indicates triplet excited state).

In vivo bilirubin is largely associated with albumin. The albumin molecule contains unsaturated amino acid residues that react with singlet oxygen, and that in the presence of bilirubin, could compete with the pigment for singlet oxygen.¹⁵⁶ Hence, the photooxidation of bilirubin in serum or in aqueous solutions containing albumin is likely to be slower than it would be in the absence of the protein. Under these conditions, the radical-involving process may assume greater importance.

When aqueous solutions of bilirubin are exposed to air or oxygen in the

dark, the pigment slowly disappears and is converted predominantly into colourless products. If the reaction is followed by spectrophotometry, a sequence of change is observed similar to the sequence observed when bilirubin is photooxidized. However, this reaction is completely inhibited by the presence of albumin, and so is the isomerisation into bilirubin III α and bilirubin XIII α .

Aims of the Project

Bilirubin is a powerful metabolic poison when used in a variety of in vitro systems, but the basis of pigment neurotoxicity in vivo is not understood. Therapy is directed towards preventing accumulation of bilirubin in excess of the infants' plasma binding capacity and toward rendering the pigment nontoxic and unable to penetrate the blood-brain barrier.

It must be mentioned that the pigment does not have any well-defined physiological role, however, its association with jaundice has interested numerous biochemists, chemists and pharmacologists. Thus, bilirubin can be regarded as a probe for studying transport and biotransformation of many other non-polar compounds. It also enjoys a continued physiological interest because it is such an obvious solute in bile and shares the same or similar "carriers" for hepatic uptake and biliary transport with many other organic anions.

The chemistry of bilirubin and its congeners has, therefore, aroused interests amongst organic, analytic, physico and photo-chemists and this may possibly lead to a clear understanding of the molecular biology of these pigments.

The studies bear on two questions. How does bilirubin bind to serum albumin so as to facilitate its release for hepatic uptake? Secondly, where is the primary binding site of bilirubin on the albumin polypeptide chain?

The binding site of a ligand on a macromolecule helps to understand the existence of excess ligand in plasma which may be due to competition with other ligands.

The complete details of the photometabolism of bilirubin in the jaundiced newborn and the mechanism of phototherapy, however, is still poorly understood. Specific aspects of the problem that merit further study include the following: The identity, toxicity, and mechanism of formation of the photoproducts require elucidation, and it needs to be determined whether the initially formed photoproducts are further metabolized to secondary compounds before they are excreted. Useful information regarding these points might be obtained from in vitro studies of the photochemical reactions of bilirubin bound to skin, lipid and albumin.

Chapter 2

2:1 Introduction

Clarification of several aspects of the metabolism of both endogenous and exogenous substances requires the preparation of these substances radiolabelled to high specific activity. Cases such as hepatic uptake of a number of substances can best be followed by introducing radiolabelled metabolites.

For a long time a chemically stable, isotopically labelled preparation of bilirubin for biochemical and physiological studies of bilirubin metabolism, was desirable. Tritium gas was used for some time to label bilirubin in vitro,^{157 a,b} but this method is both non-specific and expensive. Tritium is introduced all over the bilirubin molecule (Figure 1, structure 4). This method produced the products of low specific activity which were also very unstable physiologically or not suitable.

Thus biosynthetic methods using the precursors of bilirubin were suggested using randomly labelled δ -aminolevulinic-acid ^3H (Δ -ALA - ^3H) or δ -aminolevulinic acid - 3, 5 - ^3H (Δ -ALA - 3, 5 - ^3H) and δ -aminolevulinic acid - ^{14}C ^{14. 158-163} (Figure 15, Structures 1, 2, 3, and 4 respectively). However, these early methods yielded products which were of relatively low specific activity, radiochemically unstable or physiologically unacceptable. Glycine - 2 - ^{14}C an inefficiently incorporated biosynthetic precursor of the heme pigments was the first method to be employed to obtain ^{14}C - bilirubin. By improving the positions of tritium on the precursor (δ -aminolevulinic acid - 2, 3 - ^3H , (Δ -ALA)) (Figure 15, structure 2) resulted in labelled bilirubin with all tritium atoms in stable side chain positions and a specific activity many times

greater than the previous products.¹⁶⁴

Obviously, most of these methods have their disadvantages as can be said of the low yields and low specific activities which are compounded with lack of stability of the radiobilirubin. Hence, it was open to investigators to think of new in vitro procedures of obtaining radiolabelled bilirubin. As is the case with research, a simple method free of limitations such as low intensity of labelling, cost of the precursor materials and laboriousness was reported.¹⁶⁵ (Figure 16). The tritium in the form of tritiated water in chloroform in the presence of thioacetic acid adds to the exo-vinyl group of bilirubin. The yield in this case was about 2% after pyrolysis of the adduct, with a specific radioactivity of $20\mu\text{Ci}/\text{mmole}$.

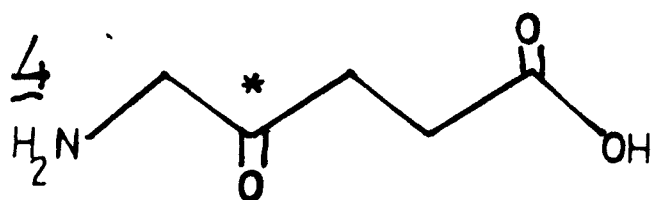
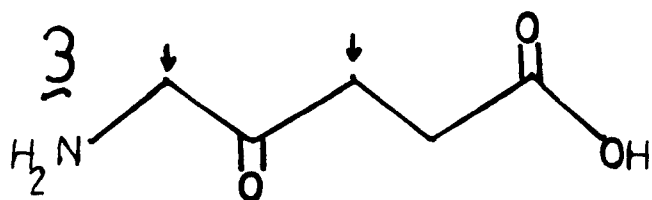
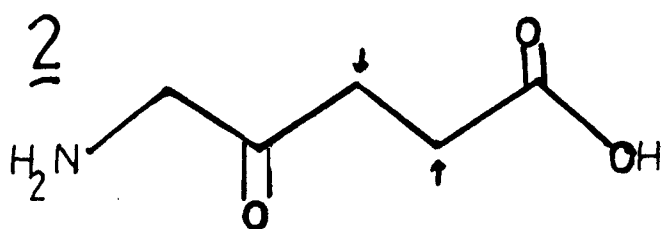
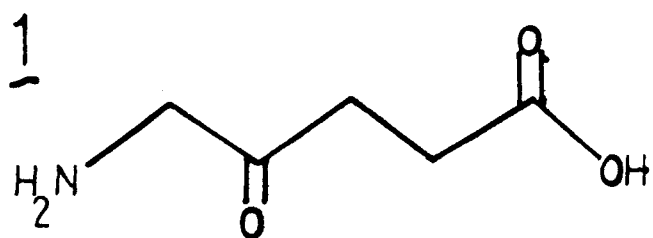
Recently, a new synthetic method of radiobilirubin was reported in our laboratory.¹⁶⁶ This method although it had a 40% yield was laborious since bilirubin dimethyl ester was the starting material. The tritium was introduced or incorporated into the methylene groups α - to the carboxyl groups in the propionic side chains.

In an attempt to improve on the latter method, a one step procedure was developed for the preparation of (^3H) - bilirubin IX - α in good yield from unlabelled bilirubin. This method is a development of a method introduced for the alkylation of aliphatic carboxylic acids.¹⁶⁷

2:2 Materials

n - Butyl-lithium, di-isopropylamine and hexamethylphosphoric triamide were obtained from Aldrich Chemical Co., Gillingham, Dorset. Bilirubin IX α was obtained from Sigma (London) Chemical Co., Kingston-upon Thames, Surrey, U.K. and was purified before use.¹⁶⁸ Tetrahydrofuran (AR.) (THF) was distilled and dried over molecular sieves before use. Silia gel plates were obtained from E. Merck,

FIGURE 15



$^3\text{H} \Rightarrow \downarrow$

$^{14}\text{C} \Rightarrow *$

δ-AMINOLEVULINIC ACID

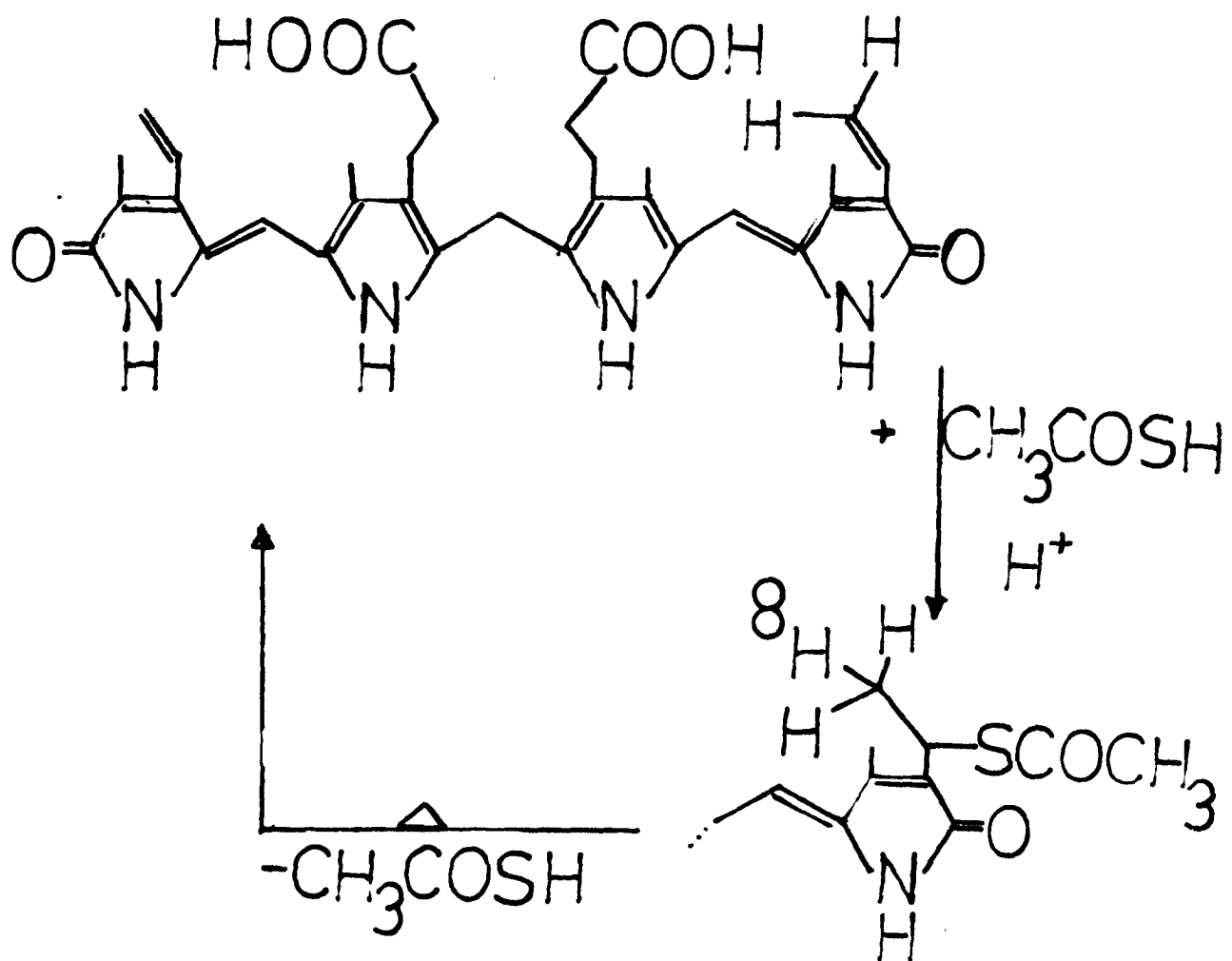
Darmstadt.

Experimental:

To a solution of di-isopropylamine (0.2g, 2.0 mmol) in anhydrous tetrahydrofuran (80 mL) under a nitrogen atmosphere was added n - butyl-lithium in hexane (1.0 mL, 1.6 M) and the mixture was stirred at -78°C for 30 minutes. Bilirubin (58.4 mg, 0.1 mmol) was added and the mixture was stirred at -78°C for 30 minutes after which hexamethylphosphoric triamide (1.5 mL, 8.55 mmol) was added. The mixture was stirred for a further 30 minutes and then trifluoro (³H) acetic acid (0.546 mL, 4.8 mmol) was added which was prepared by treating trifluoroacetic anhydride with an equimolar amount of ³H₂O (5 Ci/mL; specific radioactivity approximately 100 mCi/mmol). The reaction was stirred for 4 hours at 21°C and then acidified with 0.1M HCl in an ice bath.

The aqueous solution was extracted with chloroform three times (100 mL each), the chloroform extracts were combined, dried with sodium sulphate and evaporated to dryness in vacuo. The residual bilirubin was dissolved in chloroform (5mL) and purified by preparative t.l.c. on silica gel with chloroform/methanol/water (40:9:1, by volume) as solvent. ¹³²The yellow band (R_F 0.75) was excised and eluted from the silica with chloroform. The chloroform was removed in vacuo and the residual bilirubin was crystallised from chloroform/methanol (1:1, v/v) to yield 19.5 mg. This bilirubin was then suspended in water and brought into solution by adding sufficient concentrated aqueous ammonia (specific gravity 0.88). After 5 minutes the solution was acidified with 1 mM - hydrochloric acid and extracted with chloroform (100 mL). The chloroform was dried with sodium sulphate and evaporated in vacuo to give (³H) bilirubin. (19.3mg).

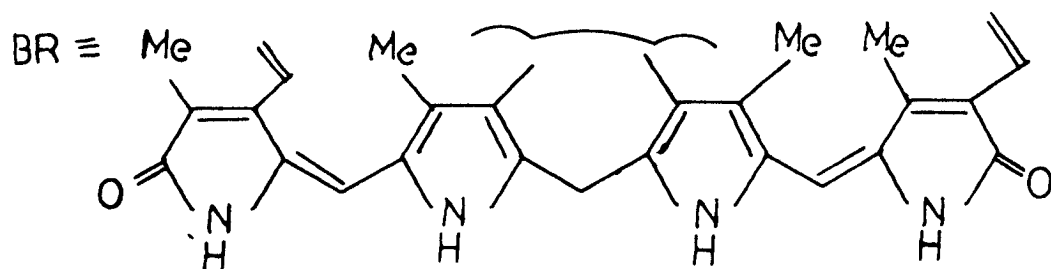
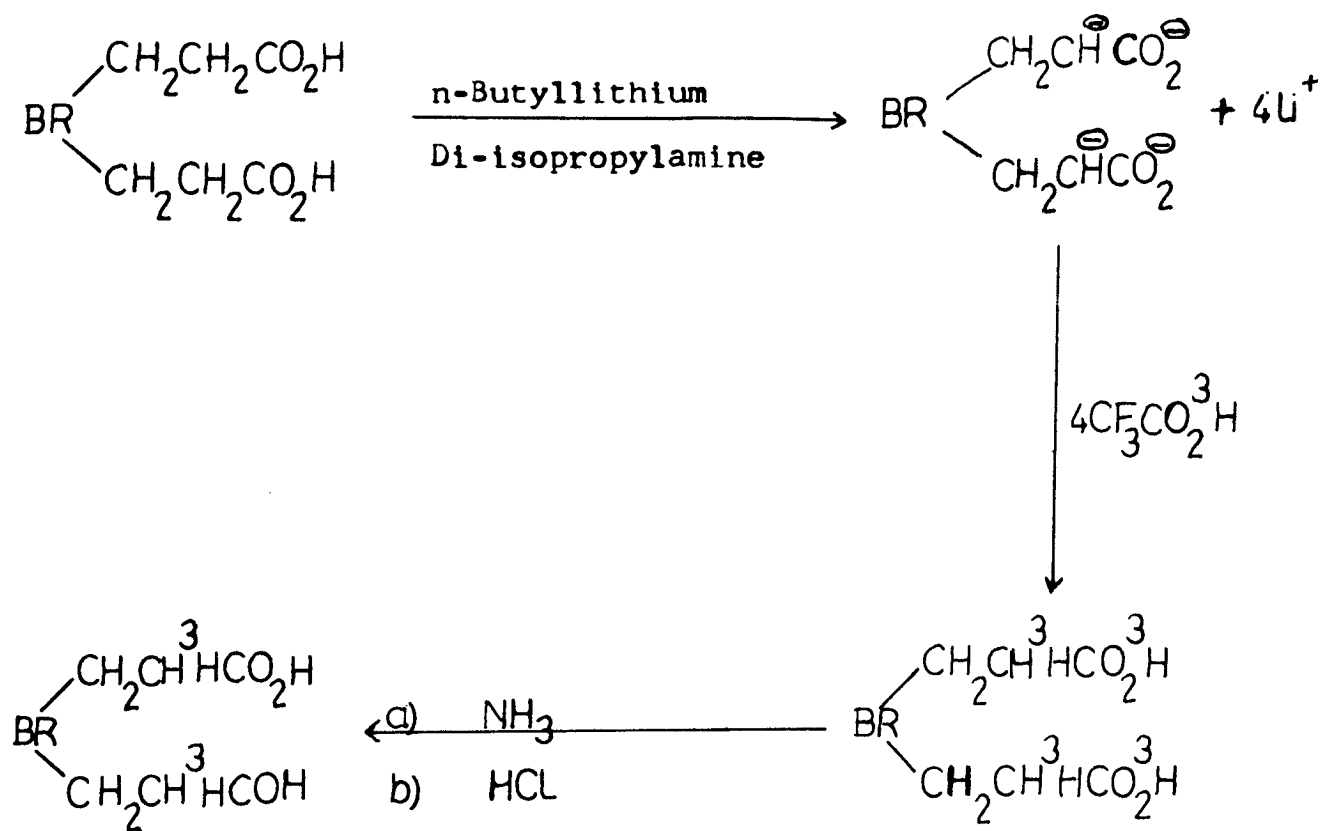
FIGURE 16



Taken from ref. (165).

Simple Procedure for Preparing (8b-³H₂) Bilirubin IX α .

FIGURE 17



Mechanism for synthesis of ^3H - Bilirubin

Table 2:1

Results :

	<u>Experiment</u>	<u>Literature</u> ¹⁶⁶
Yield (%)	33*	40
(454nm)	59,650	60,000
Specific radio activity	1.3mCi/mmol	300uCi/mmol.

* This overall yield was direct from unlabelled material unlike the indirect method through the dimethyl ester from the literature.

Discussion

The labelled bilirubin obtained was shown to be chromatographically pure¹⁴⁶ and the non-covalently bound ³H, possibly owing to the intramolecular hydrogen- bonding in bilirubin¹⁶⁹⁻¹⁷² was removed by adding to an aqueous suspension of bilirubin sufficient ammonia to obtain solutions. Immediate acidification of the aqueous solution so obtained, followed by extraction with chloroform gave a quantitative recovery of bilirubin with a constant specific radioactivity. The reaction mechanism can be formulated as follows (Figure 17). Thus comparatively high yields are obtained provided that air, light and water are excluded from the reaction. In view of the known instability of bilirubin in alkaline¹⁷³ and in acidic solutions,¹⁴⁶ it is possible that the bilirubin might isomerize under the conditions of the exchange or isolation. However, the only species that could be detected by thin layer chromatography was bilirubin - IX α . The diazotization of ³H - labelled bilirubin gave azopigments with more than 99% of the specific radioactivity of the bilirubin.¹⁷⁴ This method for the one-step preparation of (³H) bilirubin from unlabelled material is preferable to the two - step procedure involving the preparation of the bilirubin dimethyl ester¹⁶⁶ as the overall specific activity is higher

and the radioactivity of the (^3H) - bilirubin obtained is greater. By analogy with other reactions of metallated carboxylic acids,¹⁷⁵ it is assumed that only one ^3H atom was introduced into each α - methylene group of the bilirubin and no ^3H was introduced into the bridge methylene group as shown by diazotization. If placed in sealed vials and covered from light the (^3H) bilirubin did not show any appreciable loss of radioactivity for many months. Such chemical methods for the labelling of are important because the isotope is placed at a point of interest. Unlike biosynthetic methods the fate of the isotope is known in less than a day.

In the case of bilirubin biosynthetic methods are rather less specific because of the observation of the "early" and "late" labelled bilirubin. High yields of the labelled material can be used to try and avoid conventional methods for measuring small amounts of bilirubin in biological material, based on the diazo reaction, which have frequently proved unsatisfactory because of relative insensitivity at low pigment concentrations, problems encountered in standardization and loss of pigment through attachment to precipitated protein. Furthermore, labelling helps in overcoming the problem raised by tetrapyrrolic chromogens derived from bilirubin which cannot be estimated with p - dimethyl amino benzaldehyde (Ehrlich reagent).

These methodological shortcomings have hindered adequate study of several important aspects of bile pigment metabolism including: the mode of bilirubin transport in the serum at physiological concentrations, the mechanism of bilirubin exchange between serum and tissue proteins, the rate of conversion of bilirubin to non-bilirubinoid catabolites, and the nature and magnitude of the enterohepatic circulation of bile pigments. Thus isotopically labelled bilirubin of high specific activity

will help elucidate some of these problems.

Current controversies on the mechanism of phototherapy may also be clarified by following radiolabelled material. If for example photobilirubin is labelled in the propionic acid side chains, then it would be easy to follow whether its transport from the skin is carrier-mediated or not. Since several photoreactions have been reported on bilirubin, it will be interesting to observe whether labelled bilirubin injected in the Gunn rat will come in the bile in one form or another. This experiment can best be done with bilirubin labelled at specific points. Bilirubin labelled from either glycine or δ -aminolevulinic acid would not solve the problem of the competitions between photoisomerisation and photo-oxidation during phototherapy. A cleavage at the very susceptible methylene bridge would result in a possible loss of the label on this central carbon. Whilst it is true that in vivo experiments may not have a 100 per cent recovery, our labelling method i.e. tritium on propionic acid side chains would be used to answer questions such as: which is formed first, methylvinylmaleimide or hematic acid?

Elemental analysis is also necessary in biosynthetic methods least a different chromogen of similar properties has been formed. It is also known that the only way to increase the yields of biosynthetic methods is by using a bigger animal or increase the amount of the isotope. For technical and economic reasons this would be rather disadvantageous.

Chapter 3

3.1 Introduction:

In the plasma, bilirubin is reversibly bound to serum albumin and is transported to the liver where it is conjugated and then excreted. Although, many experiments have been done to locate the exact domain of albumin which binds bilirubin, no conclusions have yet been made as to the exact site of the binding. For example, affinity labelling of human albumin with bilirubin that had been activated with Woodward's reagent K (2 - ethyl-5m - sulphophenylisoxazolium hydroxide)^{123,176} indicates that two regions (residues 124 - 297 and residues 446 -547) are attached to the bilirubin. Similar experiments were done to attach bilirubin to its high-affinity site using ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride.¹⁷⁷ However, objections can be raised to the activation of bilirubin by the above reagents as they would interact initially with the carboxy groups of the bilirubin and for covalent attachment a suitably nucleophilic amino acid side chain of the albumin must be present in a favourable position in the binding site. Thus purely hydrophobic interactions between the albumin and the bilirubin are unlikely to be revealed by these methods.

The binding of bilirubin to the high-affinity has also been studied using chemically modified albumins.^{122,123} It can be concluded that at least one reactive amino group is located at the bilirubin binding site as trinitrophenylation of its group inhibited the bilirubin binding. Later experiments, however, revealed that a lysine - 240 of the human albumin is involved in the high-affinity binding site of bilirubin,¹²⁴ and also that the modification of the histidine, arginine and tyrosine residues decreased the binding affinity, whereas modification of a limited number of amino (dansylation)¹⁷⁸ and carboxy groups, the single cysteine and single tryptophan residues does not change the affinity for bilirubin.¹²²

Coupling of ligands (dyes) to albumin by sensitized photooxidation has previously been done to help in the study of the geography of the binding sites. The dye (4 - azidobenzoyl-pentagastrin) was coupled to bovine serum albumin by sensitized photooxidation.¹⁷⁹ Thus, although bilirubin is a poor sensitizer, there is a possibility of coupling it to human albumin. The imidazole rings of human serum albumin are destroyed by photooxidation in the absence of a ligand resulting in a decreased capacity for protein binding of bilirubin.¹⁸⁰ However, irradiation of albumin with ultraviolet light does not change the affinity of the protein for bilirubin.¹⁸¹ Phototherapy has been employed in a prophylactic manner to modify the degree of hyperbilirubinemia in premature infants and also to reduce bilirubin concentrations in older infants with congenital non obstructive, non-hemolytic jaundice. Despite widespread usage of phototherapy in newborn nurseries, the mechanism by which the serum bilirubin concentration is lowered is not yet known.

In vitro studies have shown that bilirubin undergoes decomposition when exposed to light of varying sources¹⁸²⁻¹⁸⁴ and so does in vivo studies.¹⁸⁵ It should be mentioned that in vivo bilirubin is strongly bound to albumin, furthermore, a visible decrease in skin coloration is observed instead of the increase expected as a result of the release of albumin bound bilirubin. Thus further investigations would be necessary to explain this disappearance. The nature and cytotoxicity of the possible photoproducts of bilirubin has warranted the study of the effect of light on bilirubin in the presence or absence of serum albumin.

Experimental

3:2 Effect of Light on bilirubin IX - α

Photodecay of bilirubin in organic solvents has been done by many investigators. In an attempt to simulate physiological conditions, the

Fig. 18. Photodecay of bilirubin in the absence of human serum albumin.

Fig. 19. Photodecay of bilirubin in the presence of human serum albumin.

FIGURES 18 & 19

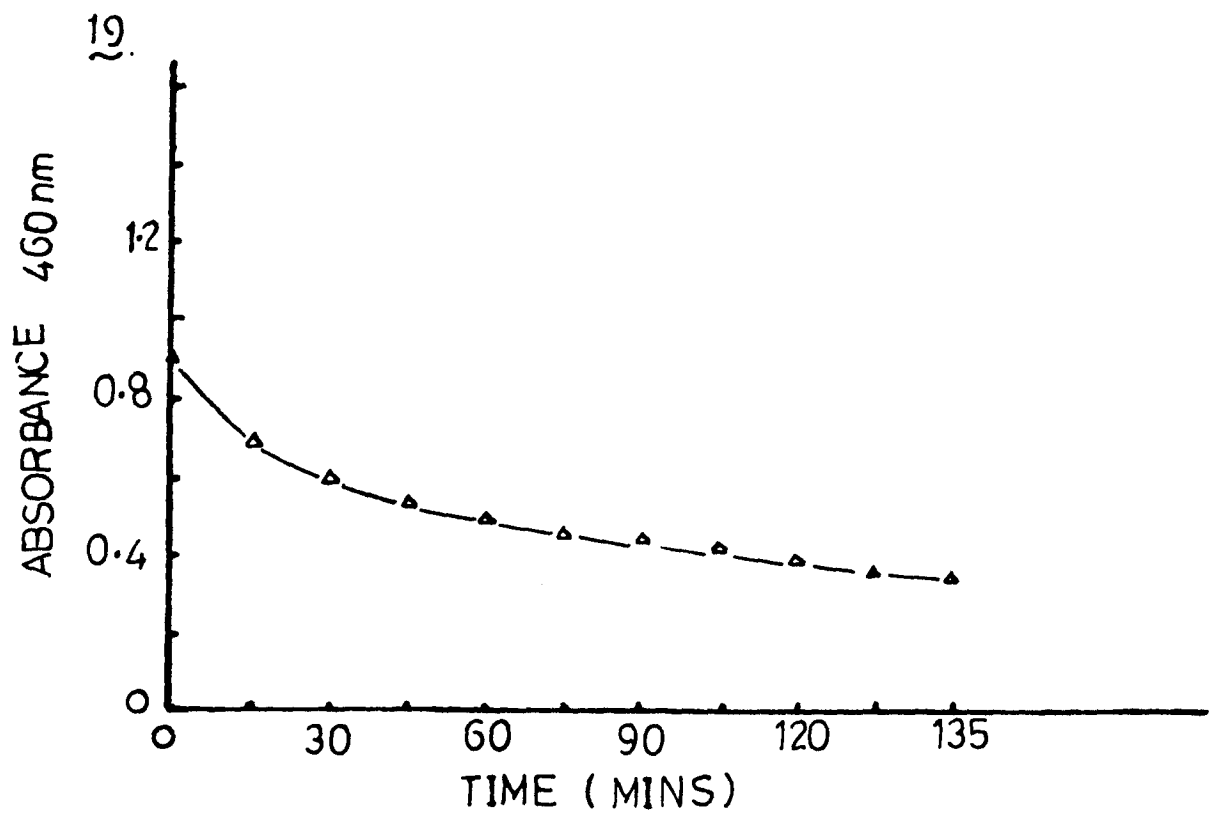
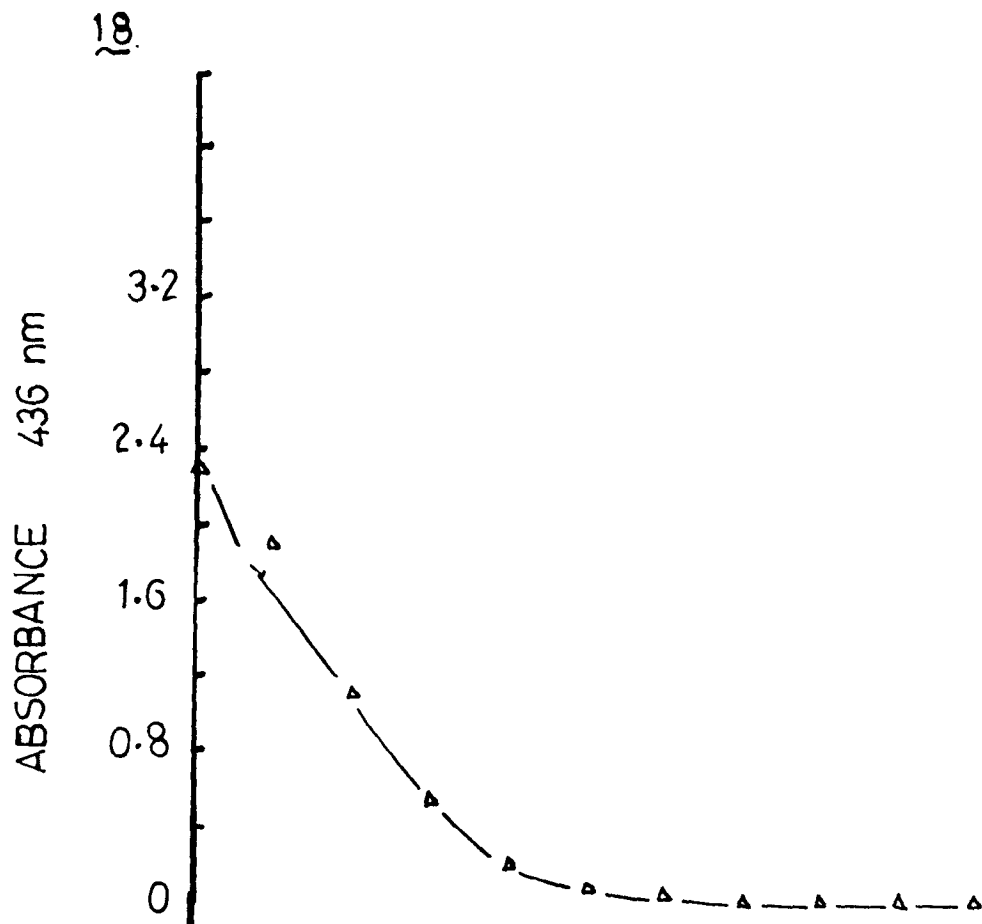


Fig. 20. Photodecay of bilirubin in the presence of human serum albumin.

($\square-\square$) using a blue filter, and ($\bullet-\bullet$), using a filter that absorbs at 325-400nm and 520-700 nm.

Fig. 21. Photodecay of bilirubin in the absence of human serum albumin ($\Delta-\Delta$), using a blue filter and ($o-o$), using a filter that absorbs at 325-400 nm and 520-700 nm.

Fig. 20

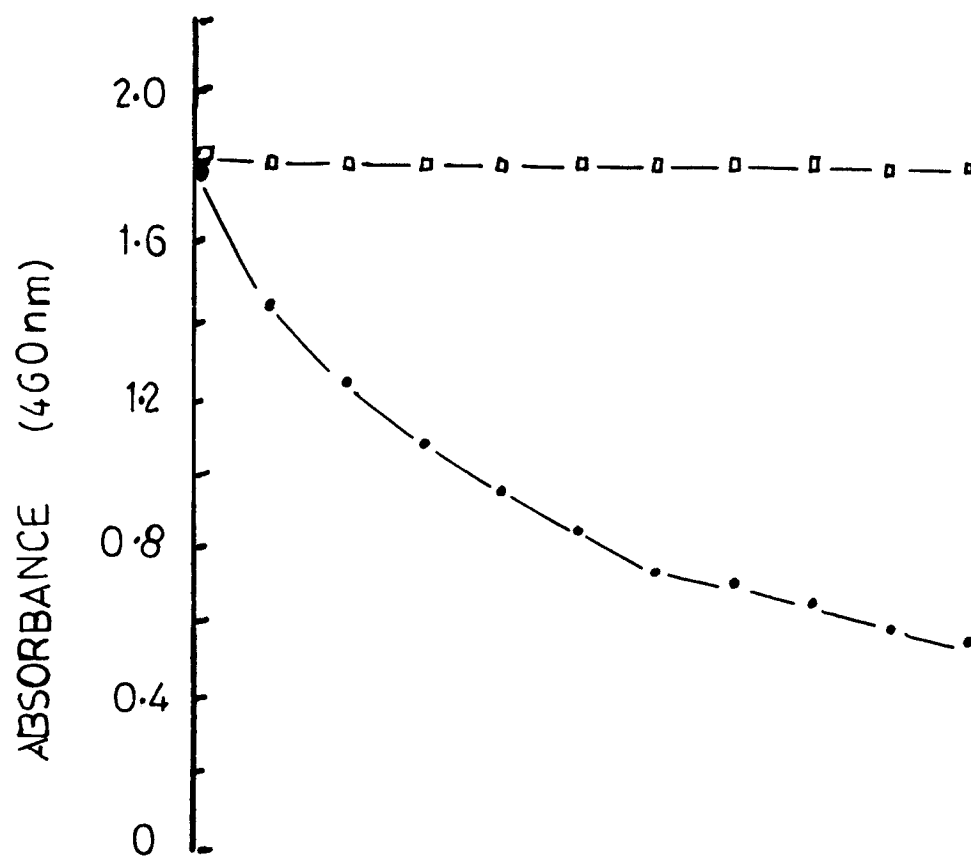
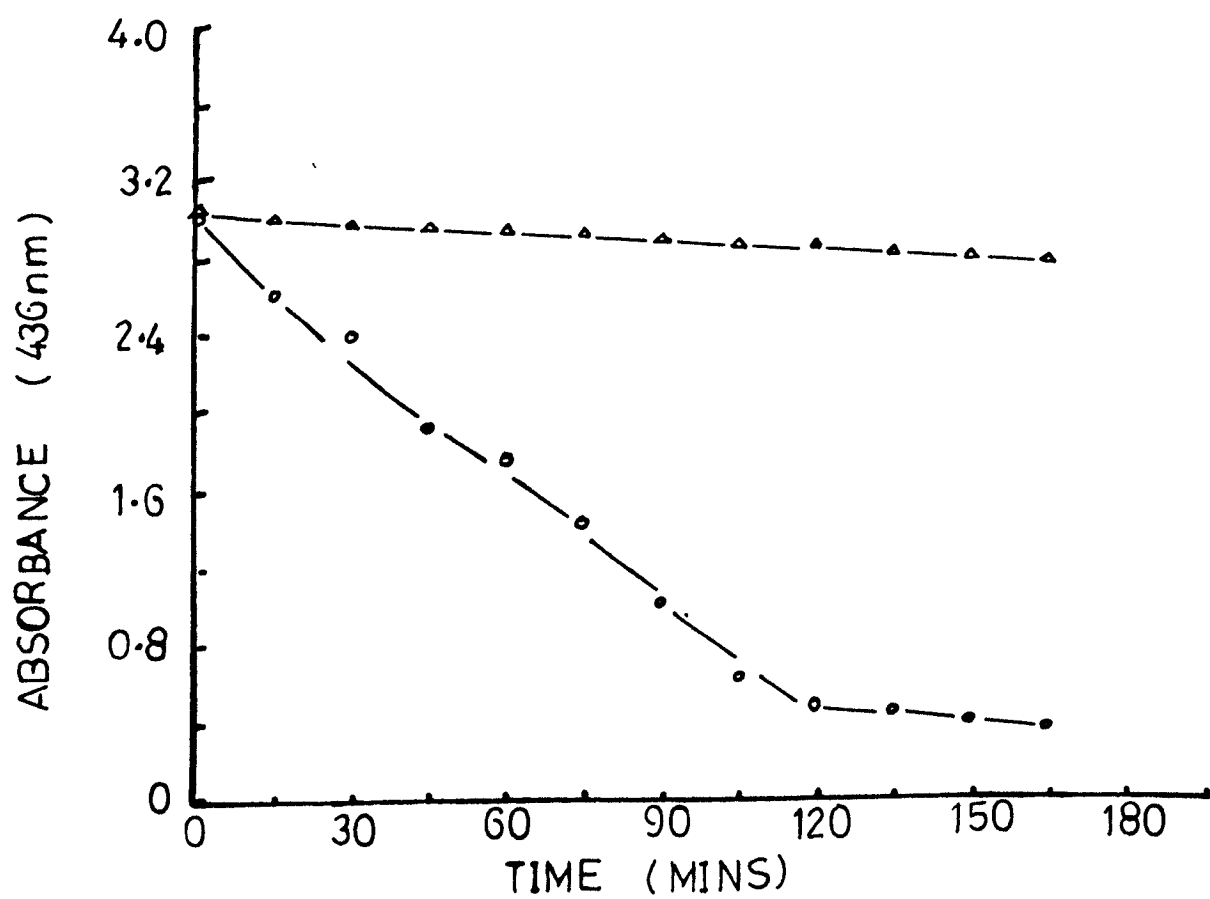


Fig. 21



photodecay was followed in the absence and presence of human serum albumin (HSA).

3:2:1. Photodecay in the absence of HSA

A solution of bilirubin ($5.13 \times 10^{-5} \text{ M}$) was made by dissolving 3mg in 0.1M sodium hydroxide (0.20 mL) and 1mM ethylenediaminetetraacetic acid (EDTA) (0.10 mL) and diluted to 100 mL with 0.10M sodium phosphate buffer, pH 7.4. Some of this solution (4.0 mL) was added to a cuvette and read on a spectrophotometer to determine the maximum wavelength.

With a maximum wavelength at 436 nm, this bilirubin solution in the cuvette was exposed to light from a lamp of 1000 W and the absorbance was read every 15 minutes for a total period of 135 minutes. Figure 18, shows the rate of decay of bilirubin in the absence of HSA.

3:2:2 Photodecay in the presence of HSA

A solution of charcoal-treated HSA ($5.13 \times 10^{-5} \text{ M}$) was made by dissolving 34.0 mg in 0.10M sodium phosphate buffer, pH 7.4 (100 mL). The bilirubin solution (5.0mL) was added to an equal volume of the HSA solution. This mixture (4.0mL) was added to a cuvette and the maximum wavelength determined as before.

The decrease in absorbance at 460 nm (λ_{max}) was followed every 15 minutes for a total period of 150 minutes as shown in Figure 19.

Photodecay of a fresh solution of the bilirubin - albumin complex was done using a filter which absorbs at 325 - 400 nm and 520 - 700 nm. The decrease in absorbance at 460nm was followed for 150 minutes (Figure 20). And Figure 21 shows the rate of decay in the absence of HSA using the filter mentioned above.

The rate of decay was also followed at respective maximum wavelengths of bilirubin - albumin complex and bilirubin using a blue filter

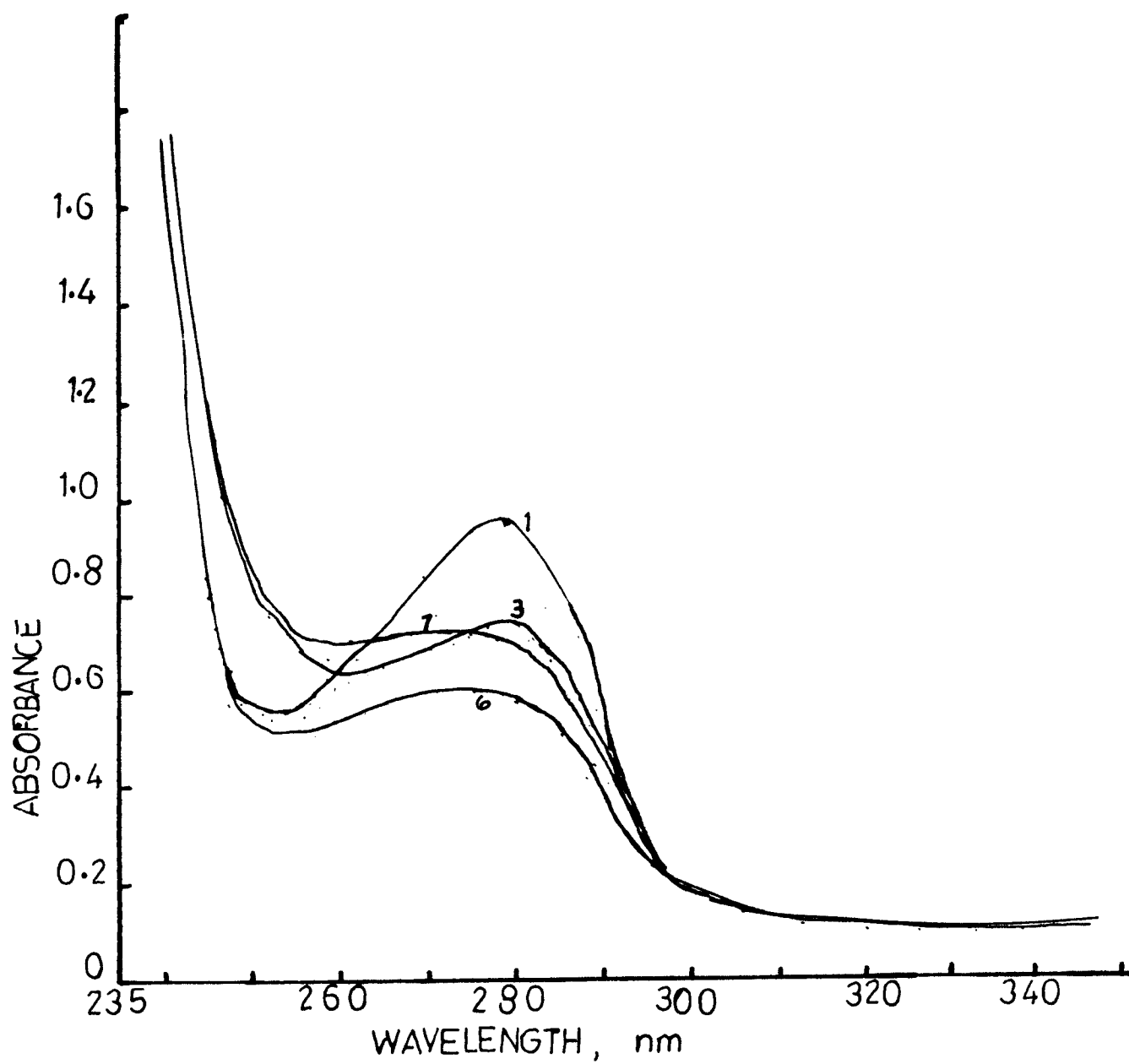


Fig. 22 SUCCINYLATION OF HSA.

NUMBERS DENOTE ALIQUOTS TAKEN
EVERY 10 MINS.

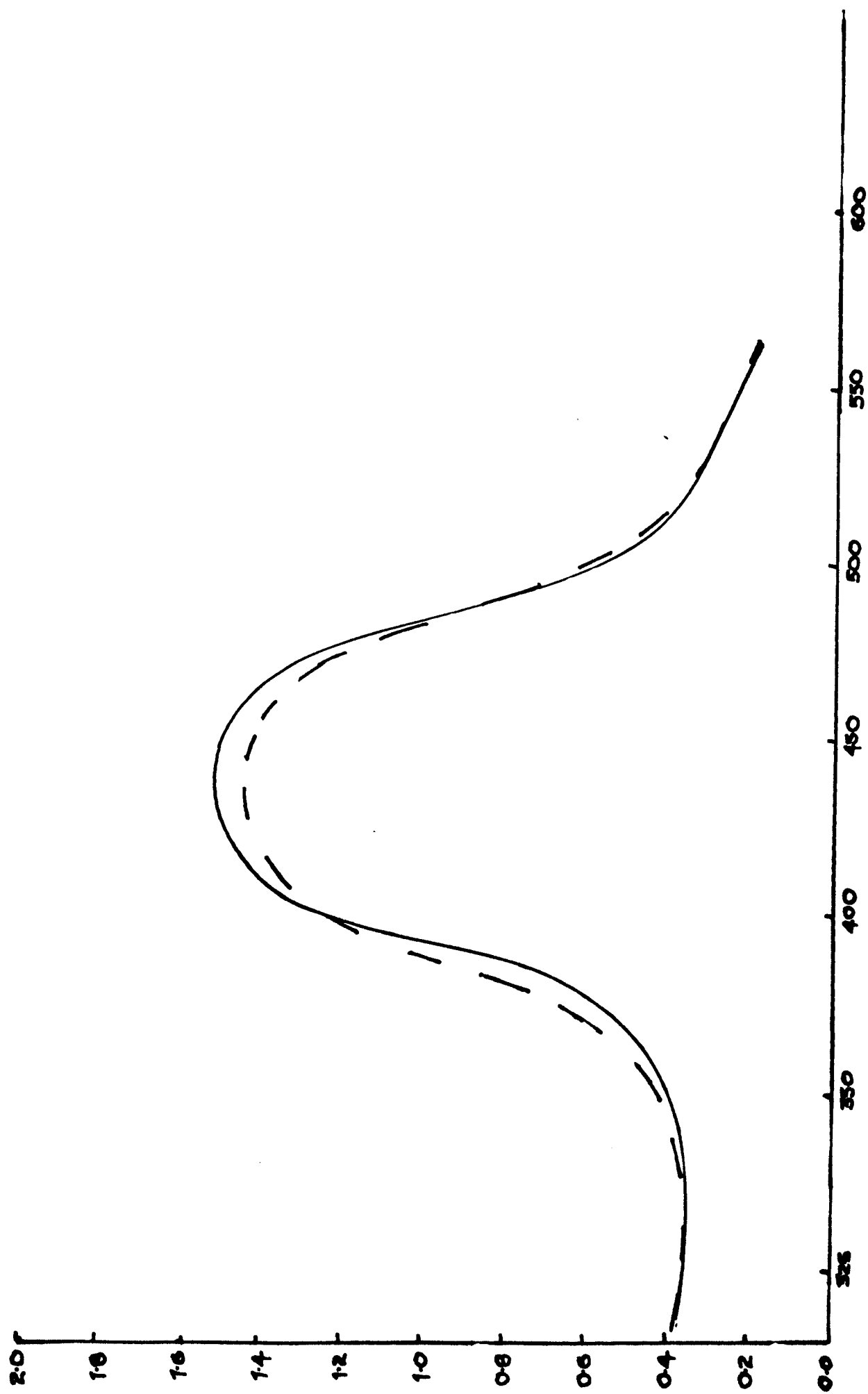


Fig. 23 ——— BILIRUBIN ALONE
----- SUCC-HSA + BILIRUBIN

(Figure 20 and 21 respectively).

3:2:3 Succinylation of human serum albumin.

When bilirubin binds to HSA, a strong bathochromic shift is observed at physiological pH (7.4). Thus in 0.1M sodium phosphate buffer pH 7.4, bilirubin has a maximum absorption at 436 nm which shifts to 460 - 462nm on binding to albumin.

Albumin was succinylated by dissolving 3.0 g in distilled water (50 mL) to which succinic anhydride (0.940 g) was added in several portions at room temperature. The pH was kept at 8.0 by adding 3.5M potassium hydroxide. After each addition, an aliquot was removed from the reaction mixture for absorption measurement in the range 350 - 250 nm (Figure 22). The reaction was allowed to proceed for one hour and kept at room temperature for 30 minutes. The mixture was then dialysed extensively against water for 36 hours at 4°C after which it was lyophilized.¹⁸⁶

Succinylation is followed by the disappearance of absorption at 278 nm of the albumin (Figure 22). Succinic anhydride reacts with ϵ -lysine, histidine, cysteine, serine, threonine and tyrosine. Thus the loss in absorbance at 278 nm is due to the reversible reaction with tyrosine residues of the albumin.

To succinylated - HSA (Succ - HSA) (5.0×10^{-5} M) in 0.1M sodium phosphate buffer, pH 7.4 (5.0mL) was added bilirubin (4.5×10^{-5} M) and 1.0×10^{-4} M), (5.0mL) in each case. The spectrum of each mixture (4.0mL) was taken on an SP 800 UV spectrophotometer immediately after mixing as shown in Figure 23.

3:3. Photo-affinity labelling of Human Serum Albumin with (3 H) - Bilirubin.

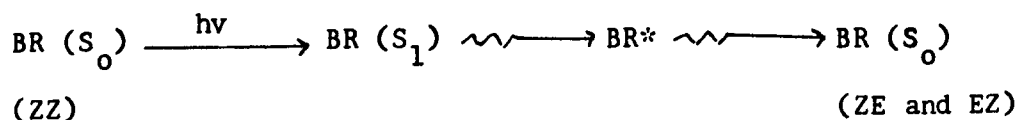
Photooxidation of proteins in the presence of sensitizers, such as

rose bengal or methylene blue, generally involves a singlet - oxygen attack on histidine residue.¹⁸⁷ Also the naturally occurring pigment, bilirubin, is able to transfer excitation energy to molecular oxygen.^{188,189} Extensive use of phototherapy in newborn infants with hyperbilirubinemia has stimulated research in this field and the question has been raised whether bilirubin is photooxidized in the skin during phototherapy, and whether it also sensitizes photooxidation of the serum albumin to which it is bound in the blood plasma.^{180,190} Reports in literature vary greatly as to the "correct" time the bilirubin should be irradiated as a result the photoproducts thus reported reveal similar variations. However, the photoproducts do not increase after 24 hours of irradiation (pH 7.8) neither do they have any inhibitory effect on mitochondrial respiration especially with succinate as the substrate. Photodegradation products may also bind to albumin, in which case the effect on mitochondrial function would be diminished.¹⁹⁰ Thus to alleviate the possibilities of the cytotoxicity of the photoproducts the time of irradiation is of importance.

These in vitro experiments do not reflect completely in vivo studies since it is believed that the effect of phototherapy in the Gunn rat is to enhance the output of either bilirubin or bilirubin anion. Therefore, the major pathway of phototherapy may not involve photooxidation or photodegradation of bilirubin; instead, it may involve enhanced excretion of bilirubin IX α .¹⁹¹ This finding is supported by studies of the effect of phototherapy on jaundiced babies.¹⁹² Recent developments in this area have proposed the formation of metastable geometric isomers that are transported in blood and excreted in bile. Thus two views appear on this field of the mechanism of phototherapy. One interpretation postulates the self sensitized

photooxidation of bilirubin¹⁹³ in the skin.¹⁹⁴⁻¹⁹⁷ An alternative view involves the $ZZ \rightleftharpoons ZE$ and EZ photoisomerisation of the meso bridges¹⁹⁸⁻²⁰² to form more soluble products. ¹⁴C labelling experiments²⁰³ have yielded results capable of lending support to either mechanism in that unchanged bilirubin appears in the bile and lower molecular weight (presumably photooxidation) products appear in both bile and urine. Although the structure of the product has not yet been elucidated, the spectrum and differential absorption spectra has been reported.¹⁹⁹⁻²⁰¹ These parameters suggest that the product is a photoisomer (EZ , ZE or a mixture). Laser flash photolysis studies identified a transient decaying and a permanent product in the presence of human serum albumin, the former having a time for half reaction of about $3\mu s$.²⁰⁴ The transient is believed to be an excited state which internally rotates on decay to give a photoisomer. However, the lifetime of the transient ($3\mu s$) is for too long lived for a singlet excited state but shorter lived than the triplet.¹³³ If the transient is a triplet, its shorter lifetime in HSA may be due to a quenching reaction with histidine or tryptophan residues in the protein. This explanation would be consistent with the observed destruction of these residues following irradiation of bilirubin-human serum albumin mixtures.²⁰⁵ However, explanation cannot be found as to why this species is insensitive to the presence of oxygen or iodide ion; possibly the bilirubin is protected by the protein.

All this does not give or explain the mode of formation of the transient and product. A possible scheme as suggested²⁰⁴ may be the following:



where BR^* may be a triplet state.

Since bilirubin may well be internally hydrogen bonded in the hydrophobic cleft of the protein, the activation energy could be made from the contributions of 38 kJ^{172} for the rupture of three hydrogen bonds plus about 10-20 kJ for an excited state rotation of a pyrrole unit about the meso bridge.²⁰⁶ Although on steric grounds a Z to E photoisomerisation is quite acceptable, the photoproducts should not exhibit any π - delocalisation over the bridging single bonds adjacent to the isomerised double bond.²⁰⁷ As bilirubin contains two oxodipyrromethene moieties, three photoproducts of this type are possible, viz. Z-E, E-Z, E-E. If the colour of bilirubin is dependent on the π - delocalisation then the last of these photoproducts should have very different spectral properties from the others.

3:3:1 Time-course for the covalent binding of (^3H) - bilirubin to HSA.

A solution of (^3H) - bilirubin (65 μM) was prepared by dissolving 3.8 mg (sp. radioactivity $1.3 \times 10^9 \text{ cpm/mmol.}$) in 0.10M - NaOH (0.2 mL) and diluting to 100 mL with 0.10M - sodium phosphate buffer, pH 7.4. A mixture of this solution (5.0mL) and a solution of human serum albumin (5mL, 65 μM) in 0.10M - sodium phosphate buffer, pH 7.4 making a total of eight aliquots, were irradiated for 15, 30, 45, 60, 75, 90 and 120 minutes, at 40 cm from a tungsten lamp (150W). A similar control was placed in the dark.

Each reaction mixture was then applied to a column (0.90 cm x

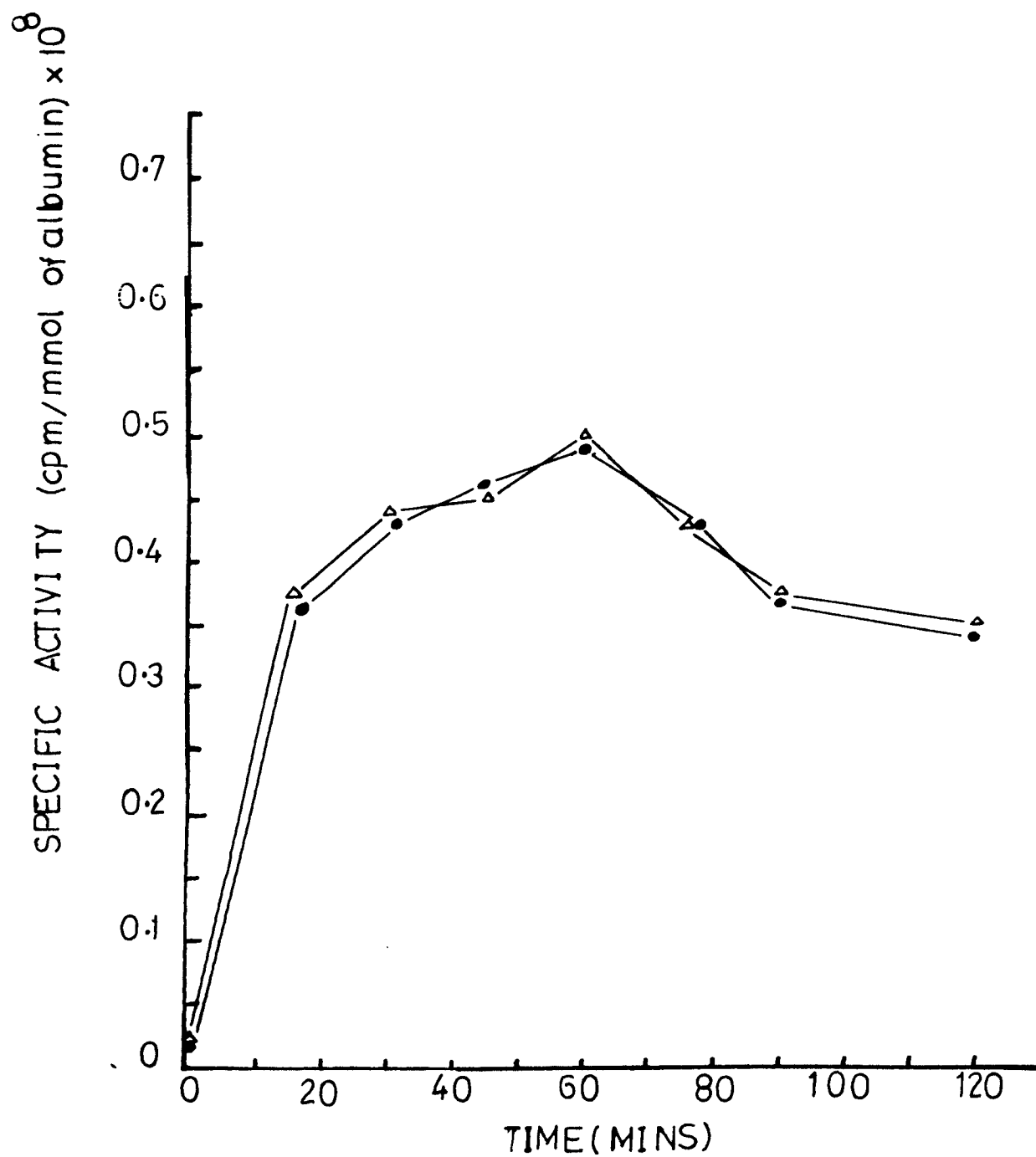


Fig. 24 TIME-COURSE OF HSA LABELLING.

●—● N₂ FLUSHED

△—△ CONTROL

15.0 cm) of Sepharose-albumin in 0.05M sodium acetate, pH 4.5.¹²⁶ The column eluate was dialysed against 2 litres of 12.5 mM - sodium salicylate, pH 7.4, for 48 hours and then against 2 litres of 0.10M - HCl for 24 hours after which time a non-diffusible bilirubin-albumin complex was obtained.

3:4. Degradation of the Bilirubin/Albumin Complex.

The structural features which give albumin its unique binding properties as a transport protein are not yet fully understood. To anticipate binding interactions and better to understand albumin function, a greater knowledge of binding mechanisms and the localization and specificity of ligand binding sites on albumin is required. Major advances in our understanding of these areas have occurred in the last few years since the elucidation of the primary sequence and structure of albumin. One consequence of the structure of albumin is that fragments produced by limited proteolysis retain, to a large extent, their original structural and binding properties. Through studies of the binding of ligands to such fragments, specific binding sites have now been located on the albumin molecule.

The strongest fatty acid sites appear to be located within the carboxy-terminal two-thirds of the molecule, the first site lying within the amino acid residues 377-503. A second less definite site lies between loops 5 and 6.²⁰⁸ These sites appear to display considerable structural specificity and are not available for the binding of other organic ligands. However fatty acids binding can affect the binding sites of other ligands on albumin as a result of the conformational change in the albumin molecule which they may induce on binding.²⁰⁹ Studies of bilirubin binding to proteolytic fragments,^{208, 210-212} together with

affinity labelling studies,^{124,213} have established that the primary bilirubin binding site is in the region of loop 4. Certain proteolytic fragments of the human albumin molecule (residues 1-386 and residues 49-307) were shown to bind bilirubin. L-tryptophan has been shown to be bound by fragments (residues 1-136) and (124-298),²¹⁵ with affinity studies locating the site near histidine 146, which occurs at the top of loop 3. Thus a common cavity or close binding sites of bilirubin and L-tryptophan do exist in the region of residues 124-298, and is sufficient enough to contain both ligands.

It has been demonstrated previously that neither lysine - 199²¹⁶ nor tryptophan - 214¹²² is involved in the bilirubin binding, as modification of these residues had no influence on the binding affinity. It should also be mentioned that dansylation of more than one lysine affects the binding of bilirubin. There is also experimental evidence that the location of the bilirubin binding site is 28A⁰ from the fatty acid primary binding site.

3:4:1 Experimental:

This was done according to McMenamy et al.²¹⁷

A solution of labelled human serum albumin was made by dissolving 100 mg in distilled water (1.0mL) and formic acid (4.0mL). To this mixture was added cyanogen bromide (100 mg).

The solution was allowed to react for 24 hours at 4°C. The solution was then diluted with distilled water (1:1 by vol.) and chromatographed on a column (2.5 x 40 cm) of Sephadex G - 25 (coarse) in 1% propionic acid. The protein was collected and concentrated to 5.0 mL in a Diaflo apparatus PM 10 membrane ultrafilter, Amicon Corporation at a pressure of 15 p.s.i.

The concentrated solution was fractionated on a Sephadex G - 100

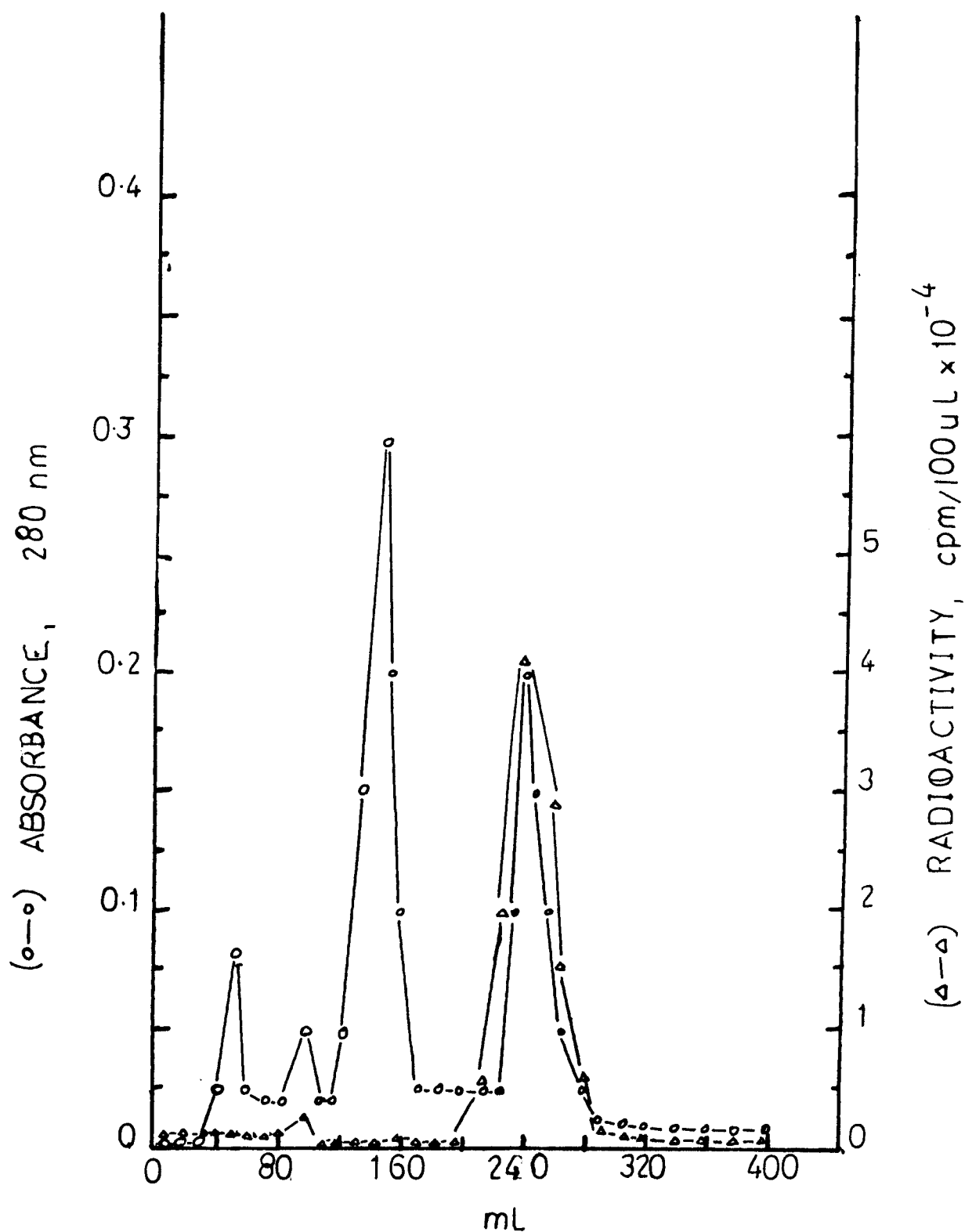
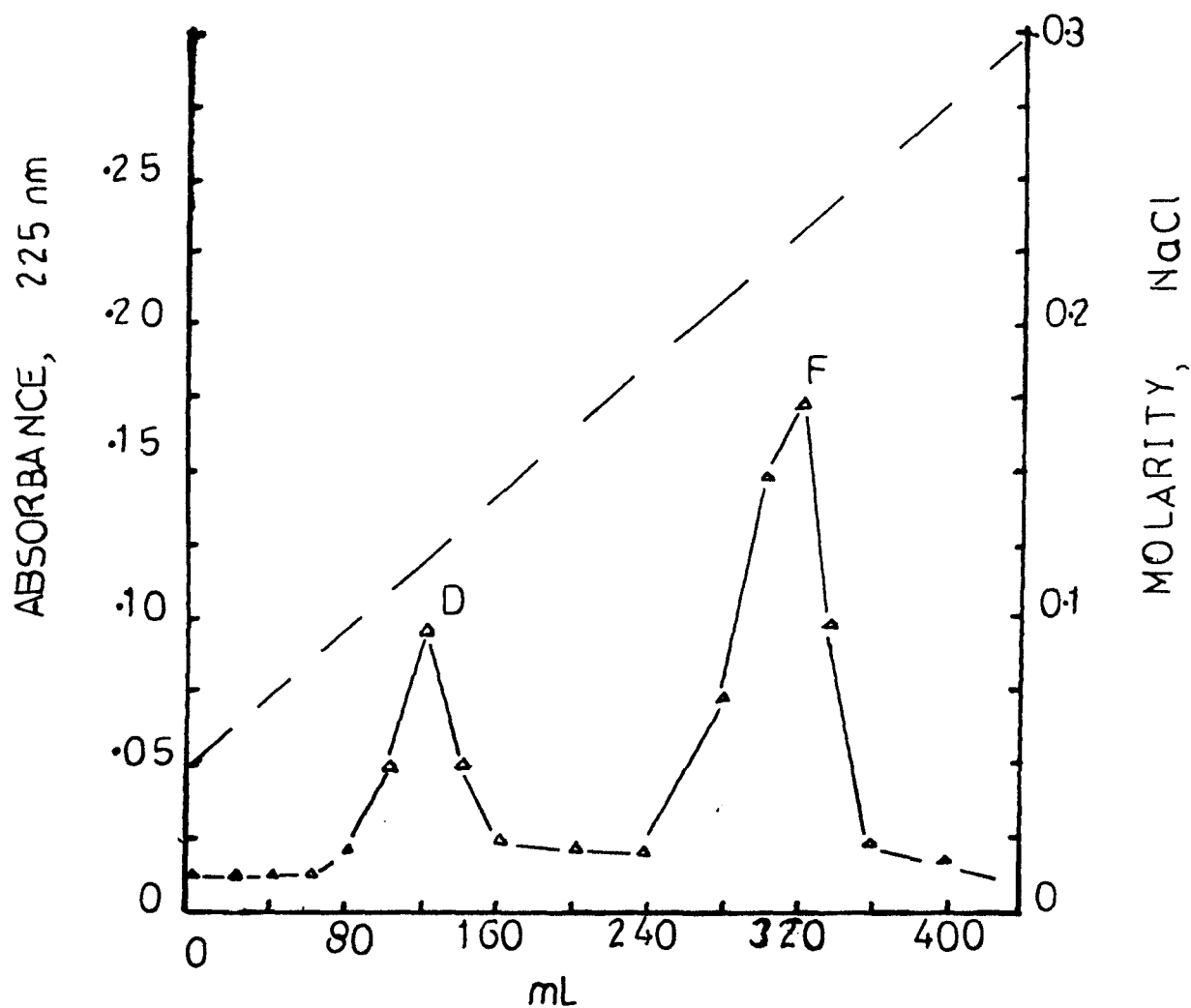


Fig. 25 Elution profile of cynogen bromide-treated human serum albumin. The albumin had been labelled with bilirubin but not reduced.

Fig. 26



Elution profile of Zone II (Fig. 25) on CM-32 Cellulose column (3.5 x 5.6 cm). The radioactivity mostly in peak F which was further treated with pepsin (see text).

column (2.0 x 90 cm) with 5% propionic acid, 0.1M NaCl at 22°C. The flow rate was 30 mL to 50 mL per hour (Figure 25). The radioactivity was determined in the fractions of the eluate and was found associated with zone II (Figure 25). Fractions from ZONE II were pooled, diluted 1:1 with distilled water concentrated as before to about 5.0mL. The pH was raised to 3.3 and then placed on a carboxymethyl-cellulose column (3.5 x 5.0 cm) (Whatman CM-32). The elution was done with 0.05 to 0.3M NaCl gradient in 0.01M PO₄, pH 2.7 at 22°C with a flow rate of 400 to 600 mL per hour (Figure 26).

The fractions pooled from peaks D and F (Figure 26) were adjusted to pH 4 to 4.5 by addition of 1M NaOH and concentrated as stated above. Radioactivity was determined by adding aliquots (100 uL) of each peak to a scintillant Toluene/ethoxyethanol/PP0/POP0P. Protein concentration was determined by the method of Lowry et al²¹⁸ and the material was subsequently lyophilized.

3:4:2. N-terminal analysis:

The N-terminal amino acid for the peptides were determined following the procedure of Gray.²¹⁹ After oxidation with performic acid, the peptide (0.3mg) was dissolved in 50uL 1% SDS in a small pyrex test tube and 50 uL N-ethylmorpholine was added. A freshly made solution of dansyl chloride (25 mg/mL, 75 uL) in anhydrous dimethyl formamide was added and mixed; the reaction was allowed to proceed at room temperature for 3 hours after which the labelled peptide was precipitated by adding acetone (0.6mL) to the reaction mixture. The resulting precipitate was compressed by centrifugation, washed with 80% acetone (500 uL) centrifuged again, dried by evaporation in vacuo and hydrolysed with 6 N HCl (150 uL) at 110°C for 18 hours. The resulting dansyl amino acid was identified chromatographically by

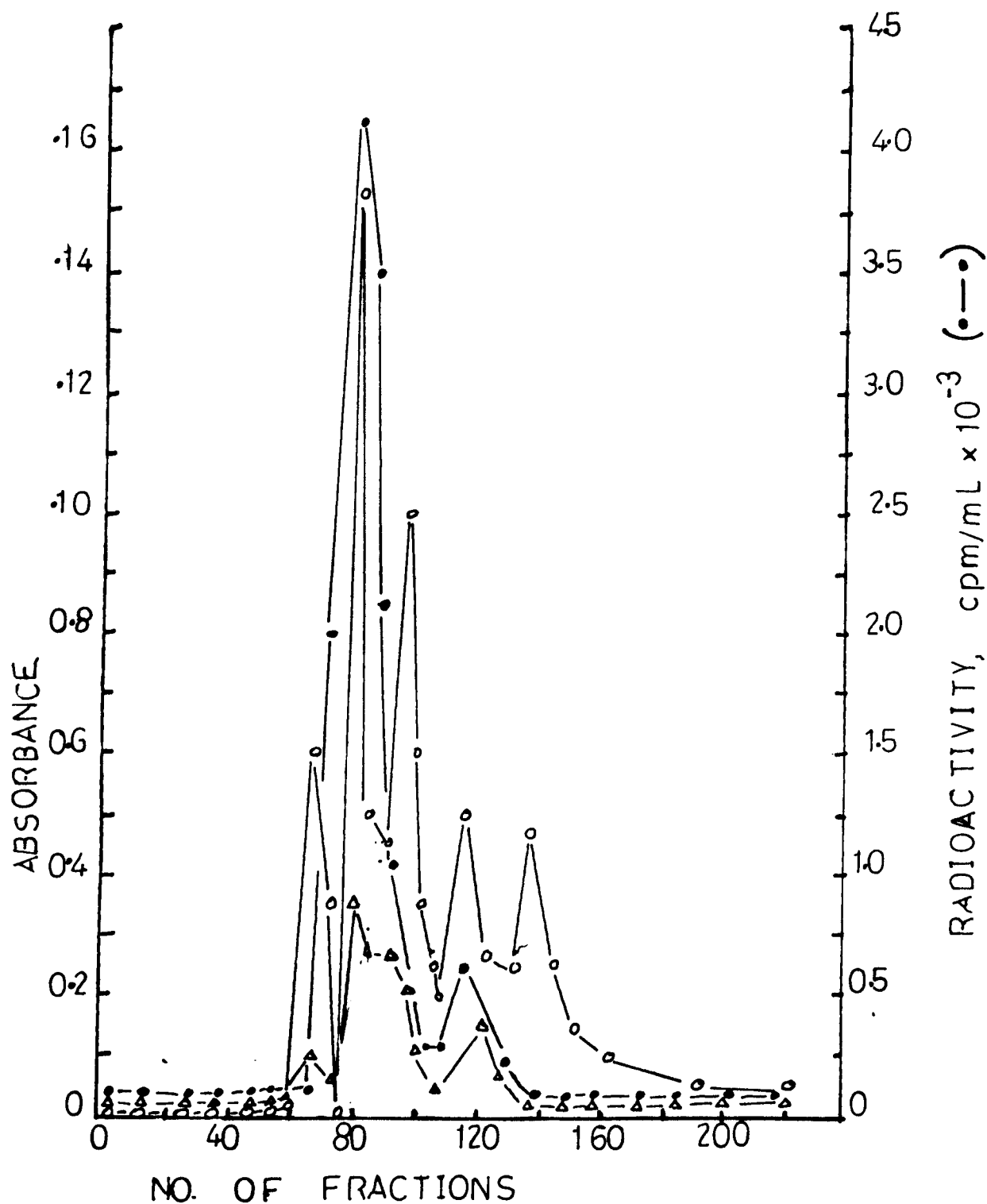


Fig. 27 Elution profile of pepsin-treated peptide F on Sephadex G-10 column (0.6 x 60 cm) eluted with 50 mM sodium phosphate buffer, pH 7.70 containing 0.15 M NaCl. The flow rate was 12 mL per hour.
 (○—○) absorbance at 225 nm and (△—△) absorbance at 280 nm.

comparison with dansyl amino acid markers on polyamide layer sheets employing the solvent systems of Hartley.²²⁰ The dansylated amino acid was visualised under ultraviolet light and the position of the spot corresponding to the dansyl aspartate and cysteine markers coincided with that of the spots arising from the hydrolytic products of the dansylated peptides. Aspartic acid and cysteine were therefore judged to be the N - terminal amino acids of peptides D and F respectively.

3:4:3. Treatment of peptide F. with pepsin.

The method employed was according to Franglen and Swaniker.²²¹

The peptide (10 mg) was dissolved in 1.5 mL of distilled water and the pH was adjusted to 2.45 with 1M - HCl. To this was added pepsin (0.033% w/v in 1mM - sodium acetate buffer, pH 5.40, 20 μ L) for 10 minutes at $25 \pm 0.1^\circ\text{C}$. The reaction was stopped by 1M - K_2HPO_4 until the pH was raised to 8.0. The hydrolysate was further subjected on a column (0.6 x 60 cm) of Sephadex G - 10 in 50 mM - sodium phosphate buffer, pH 7.70 containing 0.15M NaCl. Fractions were collected 2.0 to 3.0 mL and the extinction coefficients at 220 nm and 280 nm. read.

The peak associated with over 85% of the radioactivity (Figure 27) was pooled and lyophilized. A portion of it was further purified on column and the paper chromatography (descending) using n - butanol/ acetic acid/water, (5:2:3, v/v) as the solvent system. Spraying with 5% ninhydrin in n-butanol showed a single spot. The whole peptide was dialysed against 0.1M NaCl for a day and distilled water for 24 hours and subsequently lyophilized.

3:4:4 Amino Acid Analysis

The amino acid analysis was done on a purified peptide ^{from pepsin-treated} F on a Locarte automatic acid analyser. Table 3.1 shows the amino acids found in the labelled peptide. Residues are expressed as mmoles

per mg of peptide.

Table 3:1

Asp	1.8	Val	1.2
Thr	0.9	Met	0
Ser	1.8	Ileu	0.6
Glu	2.7	Leu	1.1
Pro	2.0	Tyr	0
Gly	4.4	Phe	0.7
Ala	5.0	His	0.5
Cystine	0	Lys	0.9
		Arg	0.5

Table 3:2 Residues 216-264

Asp	(5.0)
Thr	(3.0)
Ser	(2.0)
Glu	(5.0)*
Pro	(1.0)
Gly	(1.0)
Ala	(6.0)
Cystine	0
Val	(4.0)
Met	0
Ileu	(1.0)
Leu	(6.0)
Tyr	(1.0)
Phe	(2.0)
His	(2.0)
Lys	(4.0)
Arg	<u>(3.0)</u>
	<u>46.0</u>
Cysteine	3.0

The amino acid content was determined by Dr. J. E. Fox, Macromolecular Analysis Service, Birmingham University.

3:4:5. Location of the peptide in the albumin sequence.

Human albumin is provided with two unique amino acid markers, the single tryptophan residue at position 214 and the free thiol group at position 34 in the sequence.

Table 3.2:

<u>Fragment F.</u>			<u>Residues</u>			
			215-241		216-242	
			<u>M</u>	<u>B</u>	<u>M</u>	<u>B</u>
Asp	1.8	(3.6)	1.0	1.0	1.0	1.0
Thr	0.9	(1.8)	2.0	2.0	3.0	3.0
Ser	1.8	(3.6)	2.0	2.0	2.0	2.0
Glu	2.7	(5.5)	3.0*	3.0*	3.0*	3.0*
Pro	2.0	(4.0)	1.0	1.0	1.0	1.0
Gly	4.4	(8.8)	0.0	0	0	0
Ala	5.0	(10.0)	4.0	3.0	3.0	3.0
Cystine	0	(0)	0.0	0	0	0
Val	1.2	(2.4)	4.0	4.0	4.0	3.0
Met	0	(0)	0.0	0	0	0
Ileu	0.6	(1.2)	0.0	0	0	0
Leu	1.1	(2.2)	3.0	3.0	3.0	3.0
Tyr	0	(0)	0	0	0	0
Phe	0.7	(1.4)	2.0	2.0	2.0	2.0
His	0.5	(1.0)	0.0	0	1.0	1.0
Lys	0.9	(1.8)	3.0	3.0	3.0	3.0
Arg	0.5	(1.0)	2.0	2.0	2.0	2.0
Total Residues	24.1	(48.5)	27.0	26	27.0	27

NH₂ - terminal, Val

* One of the residues is Gln.

Table 3:2 shows the composition of the peptic fragment with the highest amount of radioactivity. The peptide was judged to have valine as the N - terminal residue. For comparison, the nearest compositions of Behrens et al (1975) (B)⁸⁸ and Meloun et al (1975)⁸⁹ (M) are shown. There is likely to be a high level of contamination due to Glycine.

Discussion

Effect of Light on bilirubin IX- α in the presence and absence of human serum albumin.

All experiments were done in darkness and the only source of light was the lamp. Light from the lamp decomposed bilirubin in the presence and absence of the albumin but relatively slower in the former. This protective nature of albumin at molar ratios less than unity shows that the pigment is completely bound to the high-affinity site. This photobleaching of bilirubin in vitro agrees with the studies observed in the Gunn rats.²²²

The screening of light through particular filters did not change the rate of photodecay with the exception of the blue filter which almost inhibits the process. This photodecay of bilirubin is probably oxidative, although exclusion of oxygen does not arrest photodecay in the presence or absence of albumin. Presumably, albumin itself or the vinyl side chain of bilirubin substitutes for oxygen as a hydrogen acceptor.²²² The stability of bilirubin to photodecay in the presence of HSA increases with increasing pH and less so at lower pH values.²²² This could be attributed to the binding of the bilirubin dianion at higher pH values.

Biliverdin is speculated to be an early intermediate in the photodecay of bilirubin and that it is stabilized in neutral albumin solution (pH 7.4). The proportion of photoderivatives of bilirubin in the

absence and presence of albumin is pH independent but in the latter case slowly migrating spots are observed in large quantities.²²² Although the photolysed solutions were not chromatographed in these experiments, products with the maximum wavelength towards the shorter wavelength to that of bilirubin were observed. Bilirubin is, therefore, stable under red light and in protein solutions; but EDTA alone even to the exclusion of oxygen does not protect the pigment.

Effect of succinylating human serum albumin:

Succinylated - HSA was shown spectrophotometrically not to bind bilirubin at molar ratios of 1.0:0.9 and 1.0:2.0 respectively (Figure 23). Succinic anhydride reacts with a number of albumin residues which have previously been shown to be in the vicinity of the binding site of bilirubin. Although the reaction is reversible with tyrosine, the modified albumin does not have an observable change in its binding affinity for bilirubin. Furthermore irradiation of succ - HSA/ bilirubin complex in 0.10M sodium phosphate buffer pH 7.4, does not arrest the decay of the pigment. This further confirms that lysine and histidine in particular are important in the binding of bilirubin to albumin, since the modified albumin has less or no affinity for the pigment.

The Effect of time of irradiation of albumin bilirubin complex:

In a typical experiment, after irradiation for 60 minutes a solution of albumin (5.0mg/mL) had an absorption of 0.50 at 450 nm after chromatography on Sepharose - albumin column. When the experiment was repeated with degassed solutions and with N₂ flushing during the irradiation, the residual specific radioactivity was the same (Figure 24). If the irradiation step was omitted the specific radioactivity of the albumin - bilirubin complex was 2.0×10^6 cpm/mmol of albumin. The non-diffusible albumin-bilirubin complex of the highest specific radioactivity (5×10^7

cpm/mmol of albumin) was obtained after 60 minutes, of irradiation. However, photoinduced binding was observed to occur even after 15 minutes of irradiation.

The break after 60 minutes (Figure 24) may be attributed to the decrease in the content of histidyl, tyrosyl and tryptophyl residues, hence the probably unfolding of the albumin molecule. It is likely that the integrity of at least some of these amino acid side chains is critical for maintaining the native three dimensional geometry of the albumin molecule; consequently molecular regions not closely adjacent to the bilirubin binding site became accessible to the photoreactive species. It has been shown that there is an initially preferential modification of 1.5 - 2 histidyl side chains in HSA which is connected with the well-documented spatial selectivity of dye-sensitized photoreactions,²²³ so that only those amino acid residues which are adjacent to the dye undergo photooxidation in the early stages.

Photobilirubin, which has been shown to be a geometrical isomer of bilirubin,^{202,224} is the unstable intermediate present in the serum from irradiated rats, and it also binds to serum albumin. Depending on the time of irradiation a mixture of $ZZ \gg EZ \simeq ZE > EE$ geometrical isomers of bilirubin will exist in solution, and the isolation and purification of the intermediate is made difficult by its thermal reconversion to bilirubin (ZZ). This agrees with the observations (Figure 24) that flushing with N_2 did not have an appreciable effect on the bound pigment.

The relative concentrations of albumin and bilirubin were chosen so that the high-affinity binding site in the albumin was just fully occupied. Thus it was hoped that complications due to the binding of bilirubin at high - and low - affinity sites do not arise to a significant extent. It was difficult to remove final traces of radioactive bilirubin from the albumin even when no irradiation takes place. A combination of

chromatography on an affinity column against sodium salicylate and 0.1M - HCl failed to remove the final traces of radioactivity. However, the covalent attachment of bilirubin to the albumin after irradiation could be clearly demonstrated by degradation of the albumin - bilirubin complex with cyanogen bromide (CNBr).²¹⁷ No radioactivity was associated with the degradation products unless irradiation of the complex had taken place.

Pre-irradiated albumin does appear to bind bilirubin less strongly; an observation shared by other investigators.^{181,206} Photo-oxidation of proteins in the presence of photosensitizers is well known;²²⁵ however, there is some controversy on the capacity of bilirubin to induce the photo-oxidation of serum albumin to which it is bound. Experiments in D₂O, in which singlet oxygen (¹O₂) has a longer half-life than in H₂O produced more or less similar results with specific radioactivities of 1.92 x 10⁶ cpm/mmol of albumin and 2.0 x 10⁷ cpm/mmol of albumin for non-irradiated and irradiated complexes (60 minutes) respectively. However, it has been generally assumed that the occurrence of protein photomodification is unlikely, owing to the high reactivity of bilirubin for ¹O₂ coupled with the relatively low efficiency of ¹O₂ generation by electronically excited bilirubins.^{130,189,191} This assumption is in contrast with the photodamage of various biomolecules induced by bilirubin.^{226,227} Some photoproducts of bilirubin e.g. methyvinylmaleimide photogenerate both ¹O₂ and radical species,²²⁸ which may thus often display absorption spectra overlapping with that of typical bilirubin. Moreover reaction mechanisms involving transient species other than ¹O₂ (e.g. radical intermediates) have been shown to be involved in the bilirubin photochemistry to an extent depending on bilirubin conformation and solvent polarity.^{171,229} It has also been pointed out that albumin-bound bilirubin undergoes photodegradation

even in the oxygen-free solutions.²⁰⁵ It should be mentioned that the formation of dye-protein ground state complexes prior to irradiation strongly favours the occurrence of type I (radical involving) process in photosensitized reactions.^{223,230} There is, however, growing evidence that anaerobic photoisomerization is the major mechanism for increased bilirubin turnover during phototherapy, rather than photo-oxidation.²²⁴

Degradation and amino acid analysis of the labelled pigment:

In the quest to find the mechanism of phototherapy and hence the excretion of unconjugated bilirubin and the possible 'cytotoxic' photoproducts, two major hypotheses have been put forward. The first of which is that oxygen is involved in photodegradation of bilirubin. Although a number of products such as methylvinylmaleimide have been found in in vitro studies, the protective nature of HSA in vivo does pose a question as to the feasibility of this method in phototherapy. It should not be ruled out though that bilirubin photodecomposes rather slowly in the presence of albumin, and even in oxygen-free solutions.²⁰⁵ The amino acid analysis Table 3:1 shows a decrease in the amount of amino acid residues which have previously been shown to exist in or around the bilirubin binding site. The irradiation experiments are time-dependent (Figure 24), as is shown by the break after 60 minutes, which explains a possible chemical and conformational change of the HSA. In addition to these is the decrease in the binding affinity for bilirubin by albumin. On the other hand some investigators²³¹ did not find any change with HSA after 60 minutes irradiations of its bilirubin complex with eight 20W/15 Osram fluorescence lamps. This observation has been attributed to the total light intensity used.²⁰⁵ It is believed that there is a preferential modification of 1.5 - 2.0

histidyl side chains,²²³ the importance of which has been inferred from the data of chemical¹²² and photosensitized¹⁸⁰ modification studies. It is also possible that the bilirubin photoproducts may possess photosensitizing properties for amino acids, hence can promote further modifications even when bilirubin has been degraded; since several of them are water-soluble.¹³⁹ The second, and a rather current favourite, is that bilirubin IX α has a Z configuration at the meso bridge double bonds, which undergo rapid and reversible photoisomerisation on irradiation with visible light giving Z-E, E-Z and E-E isomers. The non - Z-Z isomers of bilirubin - IX α are less lipophilic and more polar than the natural Z-Z isomer. However, this process is further clouded by the fact bilirubin - IX α photoisomerizes anaerobically in aqueous solutions simulating the natural environment, to a mixture of III, IX and XIII α isomers. The III and XIII α isomers also give the Z-E, E-Z and E-E isomers.²⁰⁰ These observations, although not made in these studies do reflect a possible mechanism of phototherapy, since in vivo studies have shown that the properties of the photobilirubin(s) are similar to those of the Z-E, E-Z and E-E isomers.^{139,202,224} It is also noteworthy that bilirubin is a dianion in the binding cleft,¹²⁹ hence little energy is required to break the remaining hydrogen bonds. There also exists a rather subtle process that is the photoaddition of bilirubin¹³⁹ which in itself would not probably help the mechanism of phototherapy and thus contradicts the observed increased turnover of bilirubin after phototherapy. Irradiated bilirubin undergoes regio-specific addition at the exo-vinyl group.²³² It is therefore possible that ^{to} photoaddition/bilirubin by some amino acid of HSA does occur. In these studies a non-diffusible bilirubin attached to HSA was observed,²³³ which is supported by the photo-induced binding observed by Rubaltelli and Jori (1979).²⁰⁵ The nucleophilicity of sulphydryl and alcoholic

functions, the former being more nucleophilic would probably have cysteine, methionine, threonine and Serine as the possible candidates. Table 3:1 shows the amino acid composition of the peptic fragment of the albumin region (residues 124-297) which was associated with radioactivity. It is therefore tempting to propose a photoaddition on to the exo-vinyl of bilirubin by the residues threonine or serine depending on the spatial selectivity for the following reasons:

a) photochemical addition of alcohols and thiol containing groups has been observed;¹³⁹

b) the endo-vinyl group is less reactive than the exo-vinyl group, thus the formation of the E-Z configurational isomer will be permitted around the 5 - meso double bonds. This is further supported by the evidence that the E-Z isomer is more mobile than the Z-E isomer; and thus reverts to the Z-Z configuration faster than the Z-E isomer.²⁰⁰ Besides photobilirubin (a mixture of E-Z and Z-E isomers) binds to serum albumin;²⁰²

c) during phototherapy, substances derived from bilirubin (but not identical or isomeric with it) are excreted in bile and urine. Although the nature of these is obscure, it is possible that they could be dipyrrolic propentdyopent - like compounds that are excreted, during treatment, in urine;^{185,234}

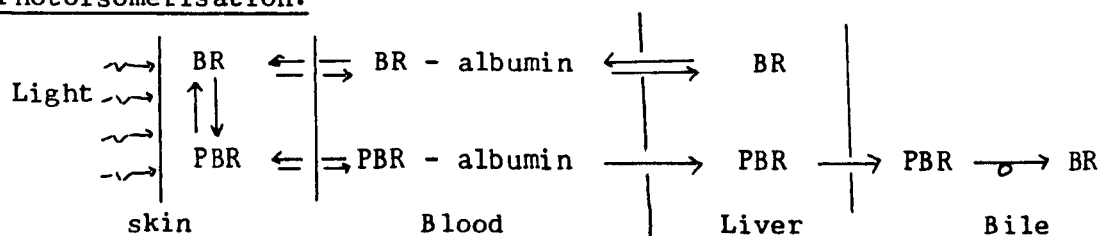
d) any feature that disrupts the usual hydrogen-bonded system and makes the tetrapyrrole more soluble in water, is sufficient to make it readily excretable.²³¹ Thus structural features of bilirubin namely an sp^3 carbon at C - 10 and two propionic acid side chains at C - 8 and C - 12 give the pigment poor excreatability compared to biliverdin - IX α .²³⁵ At least part of the serum bilirubin in animals and humans exposed to natural and artificial light is eliminated as photoadducts with nucleophilic substances, for instance GSH and, by implication, albumin.¹⁴² Obviously, albumin is appropriate to give an irreversible adduct with bilirubin;

in fact, it is well recognized that a reversible albumin-bilirubin complex^{107,236a,b} occurs in the extracellular fluids of the body.

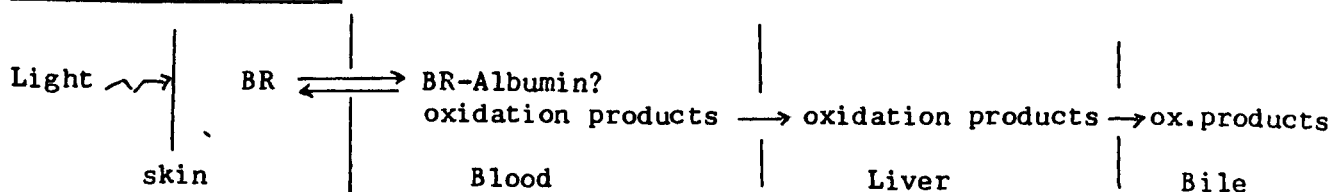
This does not in any way contradict the previous observations, but rather adds to the fact that several reactions are likely to occur during phototherapy, with the preponderance of photoisomerisation ($Z \rightarrow E$ configurational change), photo-oxidation and photoaddition. Until the nature of the photoproducts is clarified, the above processes all seem to point out towards production of more polar derivatives which are easily excreted compared to the natural pigment bilirubin - IX α . So far the following mechanisms have been proposed for phototherapy: (Figure 28).

Figure 28.

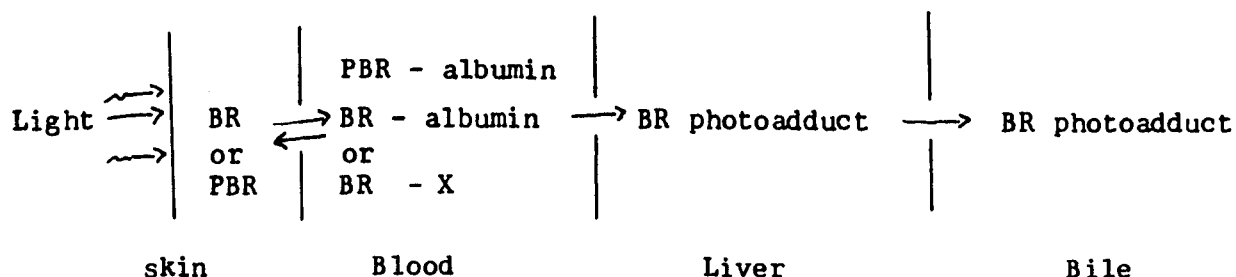
a) Photoisomerisation:²⁰²



b) Photo-oxidation:^{185,234}



c) Proposed mechanism based on photoaddition:



The bilirubin-photoadduct has to be more polar than bilirubin to facilitate

its excretion.

Figure 28: Mechanisms of bilirubin excretion during phototherapy of neonatal jaundice. (BR, 4Z, 15Z - bilirubin IX α ; PBR, photobilirubin; and oxidation products e.g. dipyrrolic propentdyopent - like compounds). X, any compound (e.g. GSH) which can be added to the bilirubin. It is important to note that photoproducts of bilirubin in the presence of HSA are different from those in the presence of BSA; the latter being identical to the CHCl_3 ones.

Location of the peptide in the albumin sequence

The N - terminal of the peptide was judged to be valine. No other sequence work was done so the tentative location was on the following reasons:

a) Lysine - 240 has been shown to be on the bilirubin binding site. The amino acid composition has an integer close to one for lysine which could possibly be the previously observed lysine.

b) Cystine, cysteine, tyrosine and isoleucine are not present. It is believed that the determined isoleucine could be leucine.

The value of glycine is very difficult to account for; it is most probably a high level of contamination. The amino acid region of the albumin was 215 - 241 or 216 - 242 compared on both known sequences by Meloun et al and Behrens et al. The region (residues 216 - 242) seems the most likely because of the presence of a single histidine which is easily destroyed during irradiation. Some amino acids have been shown to be extremely susceptible to destruction by light and hence may account for some values which do not tally. Histidine, threonine, arginine, phenylalanine, serine and tyrosine are amongst the easily destructible ones.

However, conclusive evidence can only be made if the amino acid sequence of the peptide is made. Furthermore amino acid analysis alone does not clearly show which amino acid residue(s) have been modified. Perhaps

mass spectra studies on the peptide would have further elucidated the exact nature of the modification. It will suffice to speculate that cysteine residues might have been added to bilirubin to give an adduct unknown compared to the standards used. Ideally, the amino acid sequence of the peptide shown be known since commonly used conditions, for example, constant-boiling HCl (ca. 5.7M) is used to hydrolyze a protein in vacuo at 110°C for 24 hours, the amounts of aspartic acid, serine, threonine, glutamic acid, valine, isoleucine, tyrosine, methionine, tryptophan, cysteine and cystine present in the hydrolysate may be different from the amounts in the protein (peptide) hydrolyzed.

The purity of the peptide had been judged as homogenous by end group analysis, column and paper chromatography, then dialyzed against 0.1M NaCl to displace other salts and the salt subsequently removed by dialyzing against distilled water until no chloride ions could be detected with AgNO_3 in the outer solution.

Table 3:2 shows figures in brackets which have been standardized using Arginine as the standard. The corresponding residues are given from the albumin. Many differences do exist between the peptide and the albumin especially with proline, glycine and alanine. There is no feasible explanation for the differences in proline and albumin except that they may not be involved in the binding and hence destroyed by irradiation.

Chapter 4

4:1 Introduction

In the breakdown of haem to its final products in the urine and faeces, several intermediate compounds of unknown physiological importance exist. Mesobilirubin is one of these compounds. Chemically, mesobilirubin is obtained from bilirubin by a catalytic hydrogenation of the vinyl groups to the ethyl groups. In the α series, therefore, mesobilirubins would be expected from bilirubin - III, XIII and IX α , although the IX α is likely to exist in excess over the other two. Very little, however, is known of mesobilirubin due apparently to its non-causative nature. Unlike bilirubin, it has little or no association with neonatal jaundice as a result investigators have paid little attention to the chemistry of this pigment. One reason perhaps is that isolated investigators have recorded or shown the existence of mesobilirubin in the bile,^{55,57,238} which they believed is produced extrahepatically by cellular enzymes. Using a cystine-bilirubin-peptone culture, Baumgartel⁵⁴ was able to isolate mesobilirubin and concluded that it was an intermediary product of bacterial reduction of bilirubin. It is true, however, that like bilirubin, mesobilirubin has to be conjugated before excretion.

Perhaps the most disturbing thing is that the common diazo reaction on serum bilirubin does not differ between mesobilirubin and bilirubin.⁸⁰ This method has been used to determine bilirubin in the basal ganglia in the icteric newborn. Furthermore, in vitro studies showed that bilirubin was reduced to mesobilirubin on adding basal ganglia.⁵⁹ This may suggest an enzyme catalysed reaction or further support the idea of bacterial reduction.^{56,58} In similar experiments Vogel extracted a kernicteric pigment with chloroform from the brains of infants. This pigment gave a positive diazo reaction and had a maximum absorption at 425 nm being identical in these properties

with chloroform solutions of crystalline mesobilirubin.⁸⁰ Vogel further induced experimental kernicterus by injecting solid mesobilirubin intracerebrally in newborn kittens and the pigment stained the tissue a bright canary yellow. Besides deeply jaundiced individuals have no bilirubin in their cerebral tissues.⁶⁰

A rather significant observation was that mesobilirubin binds to human serum albumin.⁸¹ Although this was a quantitative approach, it further confirms the possibility that mesobilirubin like bilirubin exists in the bile and may be transported by HSA. Fischer²³⁷ suggested that mesobilirubin may not be conjugated which if true would suggest, depending on the site of formation, that it may be more polar than bilirubin. In chloroform, bilirubin has a maximum absorption at 450nm and mesobilirubin at ca. 434 - 440 nm. Unconjugated mesobilirubin was determined in concentrations as low as 0.06mg/100mL from the serum using the chloroform extraction method.^{239,240}

Bilirubin is a powerful metabolic poison in vitro systems. Therapy is directed towards preventing accumulation of bilirubin in excess of the infants' plasma binding capacity resulting in hyperbilirubinemia and kernicterus. On the contrary no clear evidence exists to disapprove the possibility of mesobilirubin being the kernicteric pigment.²⁴¹ It is therefore necessary to determine whether mesobilirubin actually binds to human serum albumin. Labelled mesobilirubin and bilirubin were poorly excreted by Gunn rats (8%) than by normal rats (76%). In contrast mesobilirubinogen was excreted readily by both rats. This is in contrast with Fischer's²³⁷ suggestion that mesobilirubin is more polar than bilirubin. The pattern of excretion of mesobilirubin was found to be strikingly similar to that of bilirubin.²⁴² It is therefore tempting to assume that mesobilirubin and bilirubin may share the same secretory carrier. The

change of the vinyl groups to ethyl groups does not influence the "fit" of the tetrapyrrole to the secretory carrier.

Catalytic hydrogenation of bilirubin in 0.1M NaOH in the presence of palladium (Pd) catalyst was first introduced by Fischer and coworkers.²⁴³ The stepwise process of this reaction produces mesobilirubinogen as the end product. By contrast, hydrogenation of bilirubin with sodium amalgam in NaOH did not proceed in a stepwise manner, and only mesobilirubinogen was isolated.²⁴³ Another method of bilirubin reduction in methanol/ammonia in the presence of Pd/C proceeded mainly to mesobilirubin.²⁴³ Thus depending on the solvent or solvent systems various intermediates of the bilirubin reduction can be obtained.

4:2 Synthesis of mesobilirubin from bilirubin²⁴⁴

Bilirubin (200mg) was dissolved in methanol/ammonia (200 mL). This solution was then added to the suspension of a catalyst, Pd/C (10%) (200 mg). Hydrogenation was allowed to proceed for 1-2 hours at 19°C. The catalyst was filtered off and washed with the same solvent (100mL) used in the reduction. The final solution was poured into petroleum ether (2 litres) and concentrated to about 100 mL. Then sufficient chloroform (50 - 100 mL) was added for extraction. All the chloroform extracts were pooled, dried with anhydrous sodium sulphate and concentrated to about 5.0 mL. Mesobilirubin was crystallized from CHCl_3 /acetone and then from methanol/ethyl acetate.

Yield = 132.7 mg (66.4%)

λ max in chloroform = 434 - 435 nm.

M.p. = 315 - 318°C

Calculation of ϵ :

Mesobilirubin (0.6mg) was dissolved in chloroform (100 mL).

Fig. 29. U.V. spectra of mesobilirubin and albumin-mesobilirubin complexes.

1 denoted the spectrum of mesobilirubin alone in sodium phosphate buffer, pH 7.4, 0.1M.

2 denotes the spectrum of mesobilirubin-albumin complex in the molar ratio of 1:1.

3 denotes the spectrum of mesobilirubin-albumin complex in the molar ratio of 2:1.

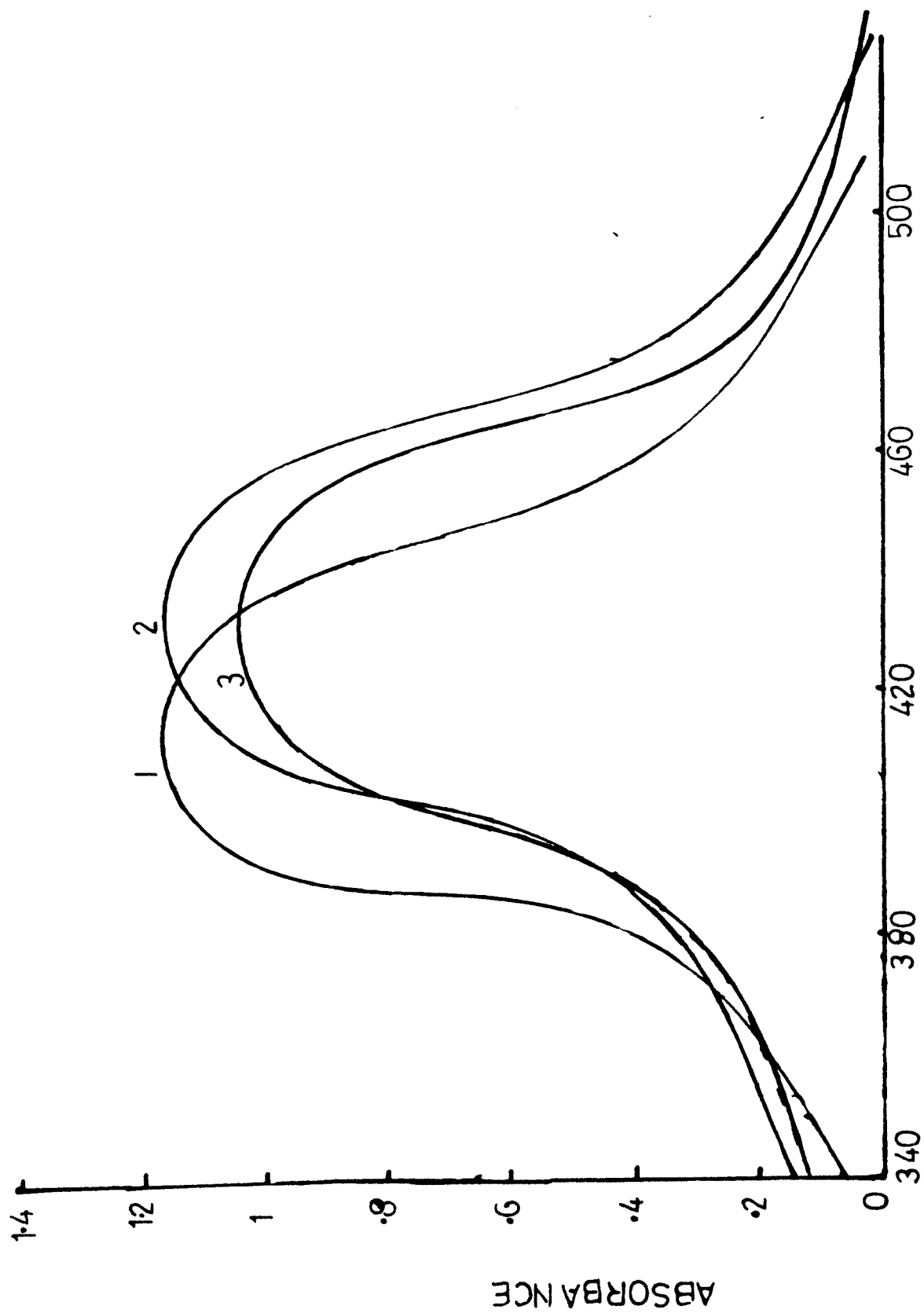


Fig.29 WAVELENGTH, nm

This solution had an absorbance of 0.58 in a cuvette of 1 cm path length.

$$A = EC l$$

$$\therefore E = \frac{A}{C l}$$

The molecular weight of mesobilirubin is assumed to be 588.7. The concentration of the solution is:

$$6\text{mg/l}$$

$$\text{i.e. } 6.0/588.7 \text{ M}$$

$$= 1.02 \times 10^{-5} \text{ M}$$

$$\text{Hence } E = \frac{0.58}{1.02 \times 10^{-5} \text{ M} \times 1\text{cm}} = 56,900 \text{ M}^{-1} \text{ cm}^{-1}$$

Table 4:1

	<u>Experiment</u>	<u>Literature</u> ²³⁹
λ_{max}	434 - 435	434 nm
E	56,900	56,000
m.p.	315 - 318°C	315°C

4:3 Binding studies of mesobilirubin to HSA

4:3:1 Spectroscopic studies:

A stock solution of mesobilirubin was made by dissolving 1.40 mg in 0.1M NaOH (0.2 mL) and diluted to 100 mL with 0.1M sodium phosphate buffer, pH 7.4. Albumin solutions were prepared to give the final molar ratios of 1.0 and 2.0 with respect to mesobilirubin. Mesobilirubin in aqueous solution, (pH 7.4), had a maximum wavelength at 410 - 412 nm; this shifted to 438 - 440 nm on addition of albumin (Figure 29). This bathochromic shift is characteristic of the binding of most ligands to macromolecules. The measurements were done on a Cecil 505 Double Beam UV Spectrophotometer using the buffer as the reference.

4:3:2. Fluorescent studies: Done according to Levine.²⁴⁵

Bilirubin alone does not fluoresce yet it does when added to albumin. The latter fluoresces due to its aromatic residue especially tryptophan. However, human serum albumin does not fluoresce very much with its single tryptophan residue compared to bovine serum albumin. Binding studies can be performed therefore either by enhancing or quenching the fluorescence of the albumin by mesobilirubin which like bilirubin does not fluoresce alone.

Experimental:

A fresh stock solution of mesobilirubin was made by dissolving 5.0 mg in 0.1 M NaOH (0.2 mL) and diluted to 5.0 mL with 0.1M sodium phosphate buffer, pH 7.4 in 0.1mM EDTA to give a 1.70×10^{-3} M solution.

Albumin stock solution was determined by measuring the absorbance at 280 nm and using $E_{1\text{cm}}^{1\%} = 5.3$. With the molecular weight of 66300, a stock solution of 1.70×10^{-4} M was made up in the same buffer.

The working solutions for mesobilirubin were 2.02×10^{-5} M and 2.028×10^{-4} M and those for albumin were 1.01×10^{-6} M and 1.014×10^{-5} M respectively. To the working mesobilirubin solutions was added albumin in 20:1 ratio before titration. This was done (a) to prevent the dilution of titrated albumin and (b) to stabilize the mesobilirubin.

Three 4mL - cuvettes were used; one filled with the buffer and the other two with albumin (2.0 mL). The latter two were arbitrary designated reference and titration cuvettes. The cuvette containing the buffer was used to set the zero or blank fluorescence, and the reference cuvette to set 1.0. The fluorescence of the titration cuvette was then read on a Perkin Elmer MPF - 3 Fluorescence

Fig. 30. Plot of observed fluorescence
against mesobilirubin-albumin ratios.
The albumin concentration was $1.01\mu\text{M}$.

Fig. 31. Plot of observed fluorescence
against mesobilirubin-albumin ratios.
The concentration of albumin was $10.1\mu\text{M}$.

The quenching effect is clearly observed at
albumin concentrations of $1.01\mu\text{M}$ than at ten
times more.

FIG. 30.

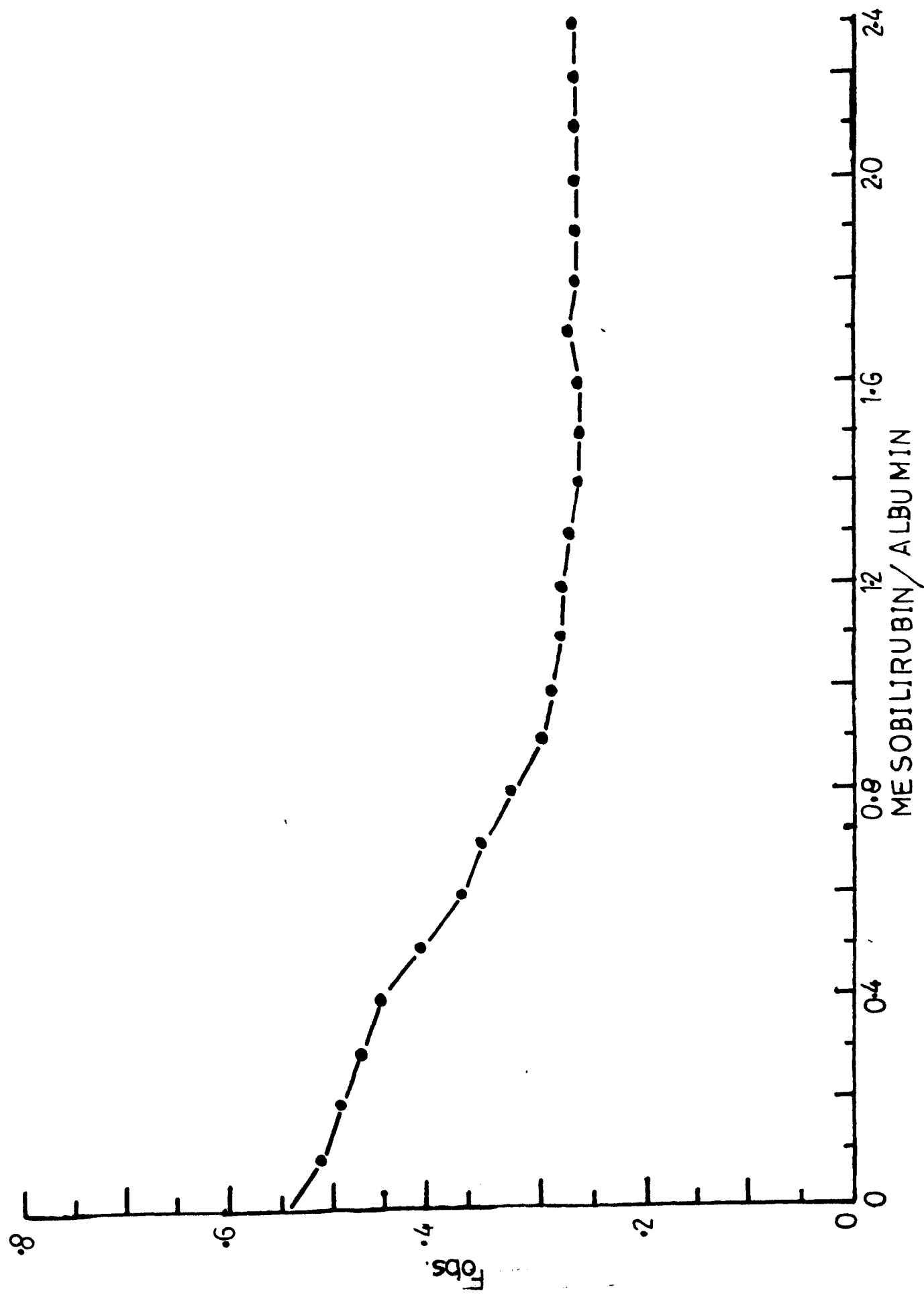
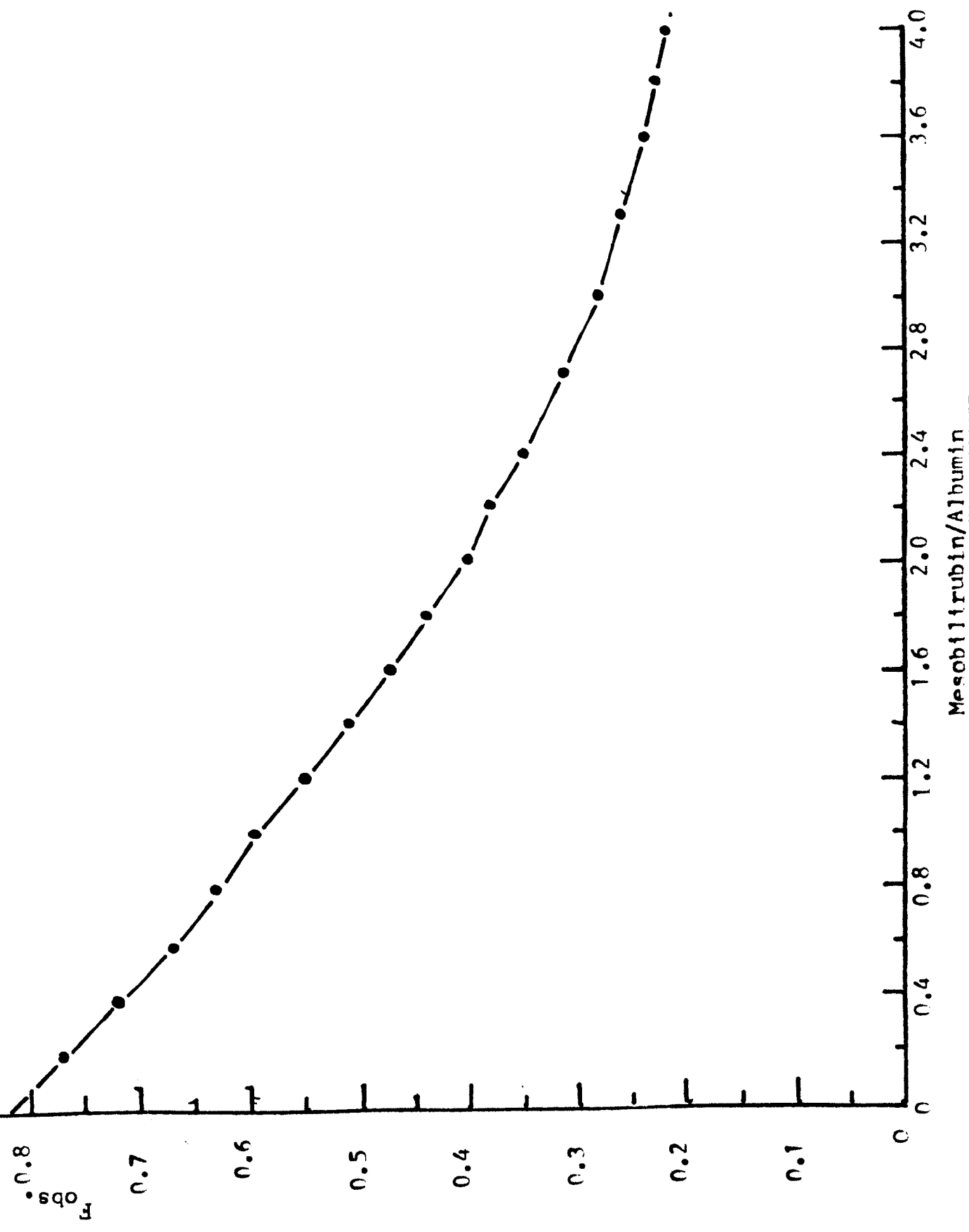


Fig. 31.



Spectrofluorimeter at about 24°C. The reference cuvette helped to check the drift in the fluoremeter response, variation in intrinsic albumin fluorescence and any fluorescence due to the buffer. Measurements were done using the different concentrations of mesobilirubin with the respective albumin concentrations.

5 or 10 μ L of the mesobilirubin solution was added to the titration cuvette with a Hamilton microsyringe. The solution was mixed by inversion of the cuvette, and fluorescence was measured. About 20 - 35 aliquots were found to be necessary in both cases. The shutter allowing light to strike the test solution was opened only when reading fluorescence. This precaution avoided significant photodecomposition of the mesobilirubin.

The excitation wavelength used was 294 nm and emission was 343 nm, with the half band width of 13 nm. A control experiment which was performed by adding the buffer to the albumin showed that all the quenching was due to the ligand (mesobilirubin). At the end of the experiment the absorbance of the titrated solution was read at 294 nm and 343 against the albumin solution. The molar absorptivity calculated from this was used to correct for the inner filter effect.

Results and Discussion

The correction due to inner filter effect was calculated as follows:

$$F_{\text{corrected}} = F_{\text{observed}} \times H$$

where

$$H = \text{antilog} \left(\frac{E_{\text{excitation}} + E_{\text{emission}}}{2} \right) \times (\text{mesobilirubin}) \left(\frac{\text{pathlength, cm}}{2} \right)$$

which becomes

$$H = \text{antilog} \left(E_{\text{excitation}} = E_{\text{emission}} \right) \times (\text{mesobilirubin}) (0.4)$$

Figures 30 and 31 show plots of fluorescence observed ($F_{\text{obs.}}$) against

Fig.32. Plot of relative fluorescence against
mesobilirubin/albumin molar ratios.
Albumin concentration 1.01uM.

Fig.33. Plot of relative concentration against
mesobilirubin/albumin molar ratios.
Albumin concentration 10.1uM

The relative fluorescence had been corrected for
inner filter effect.

Fig.32.

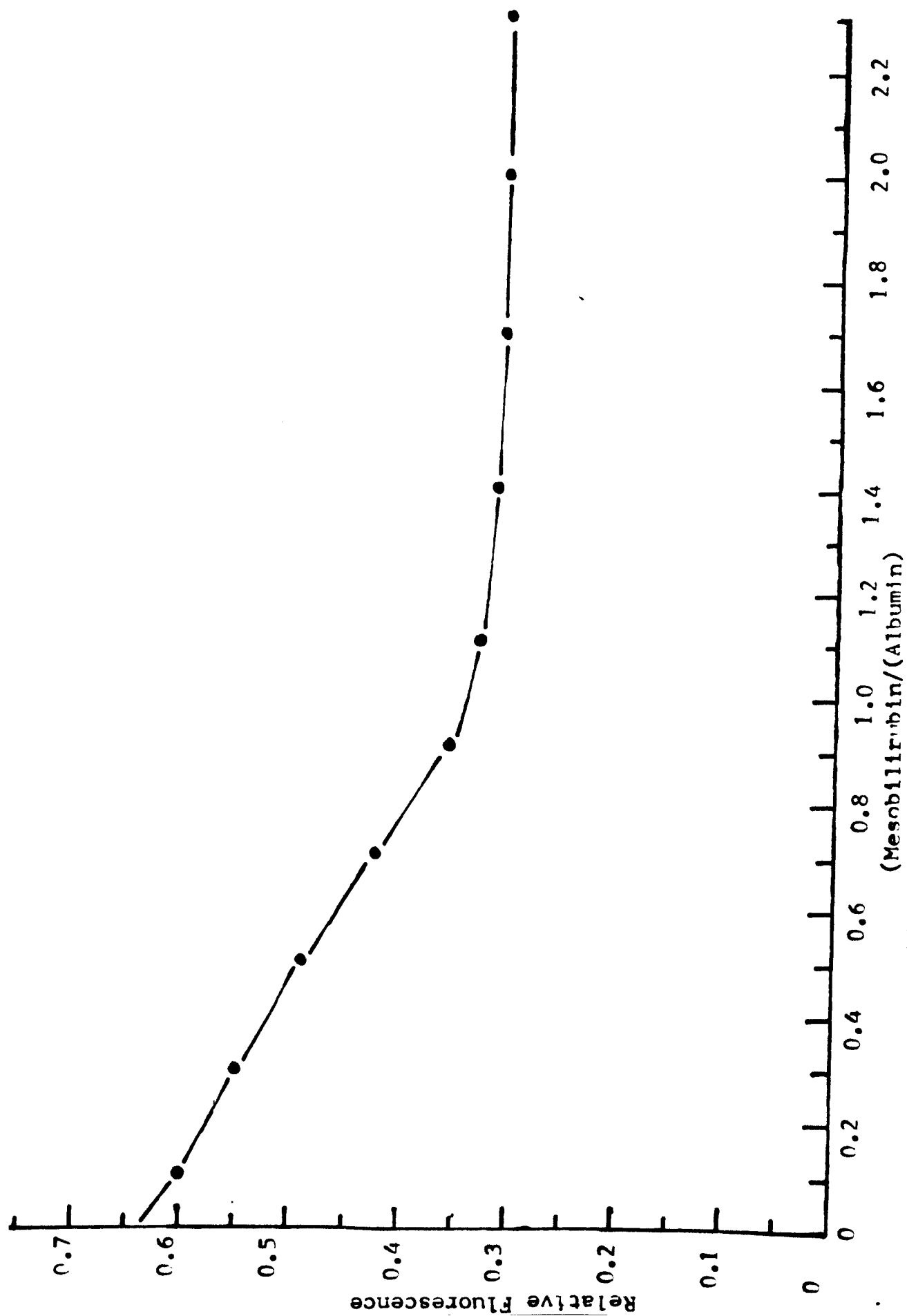
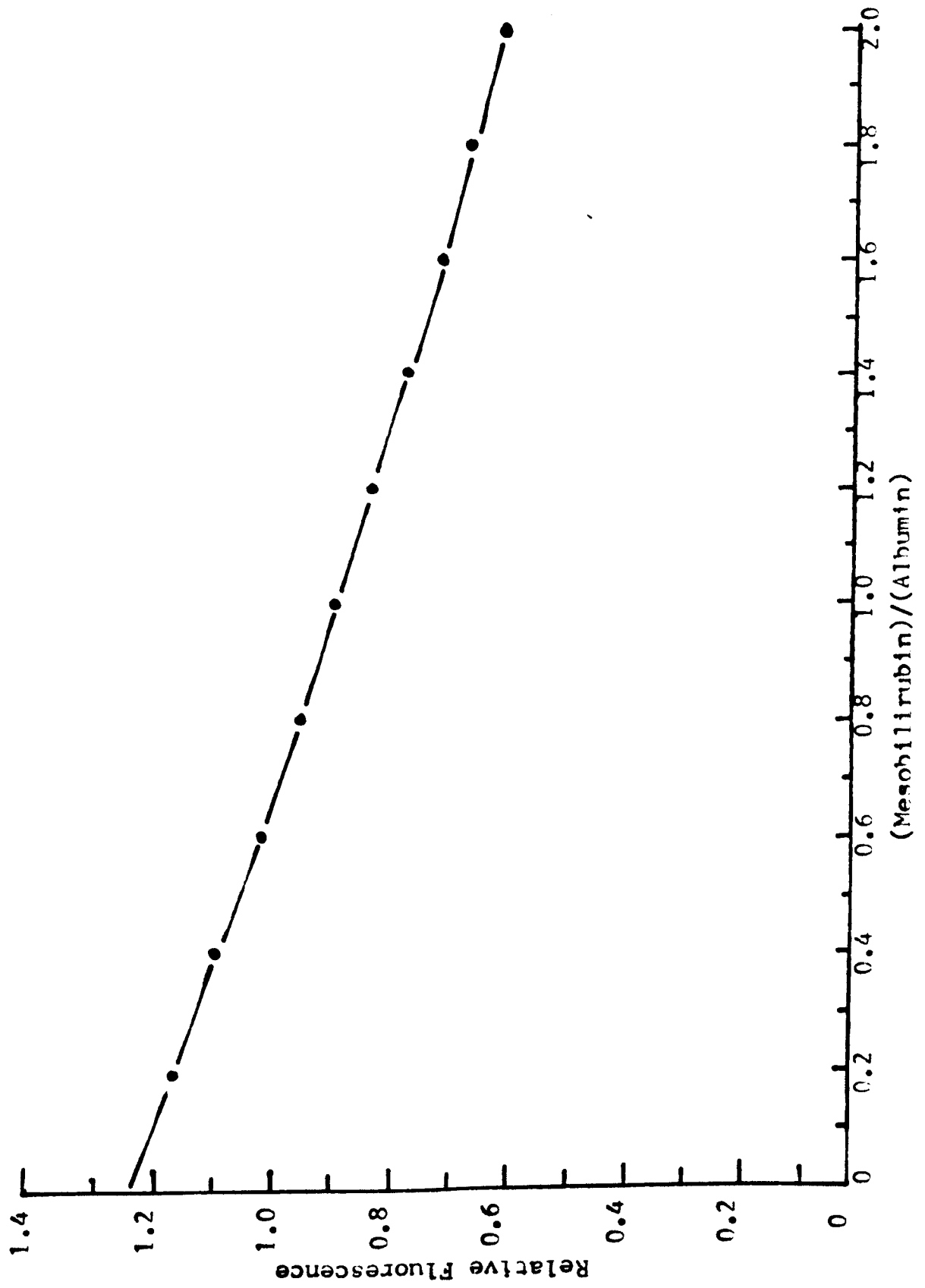


Fig. 33.



mesobilirubin/albumin ratio Q , for the 1.01 μM and 10.1 μM albumin solutions respectively. In the Figures 32 and 33 are plots showing the corrected fluorescence (F_{corr}) against the mesobilirubins/albumin ratio (Q). Plots of albumin concentrations less than 1.0 μM (not shown) were erratic perhaps due to light scattering by colloidal formation of mesobilirubin at such low albumin concentrations. The effect of the total amount of protein compared to the quenching can be observed by comparing Figures 32 and 33. When the albumin concentration was $1.01 \times 10^{-6} \text{M}$, the intrinsic fluorescence of the albumin was quenched to approximately 50% by mesobilirubin. This phenomenon is not clearly illustrated in the $1.01 \times 10^{-5} \text{M}$ solution of albumin (Figure 33). After a mole of mesobilirubin is bound, no more quenching is observed and the plot of fluorescence (F_{corr}) against the molar ratio of mesobilirubin to albumin levels off. This phenomenon is not observed in the HSA/bilirubin complexes in which the first three bilirubins to bind to HSA have the same or yield about the same fluorescence. The first mesobilirubin has this anomalous nature of quenching the fluorescence without causing a further decrease in the albumin fluorescence.

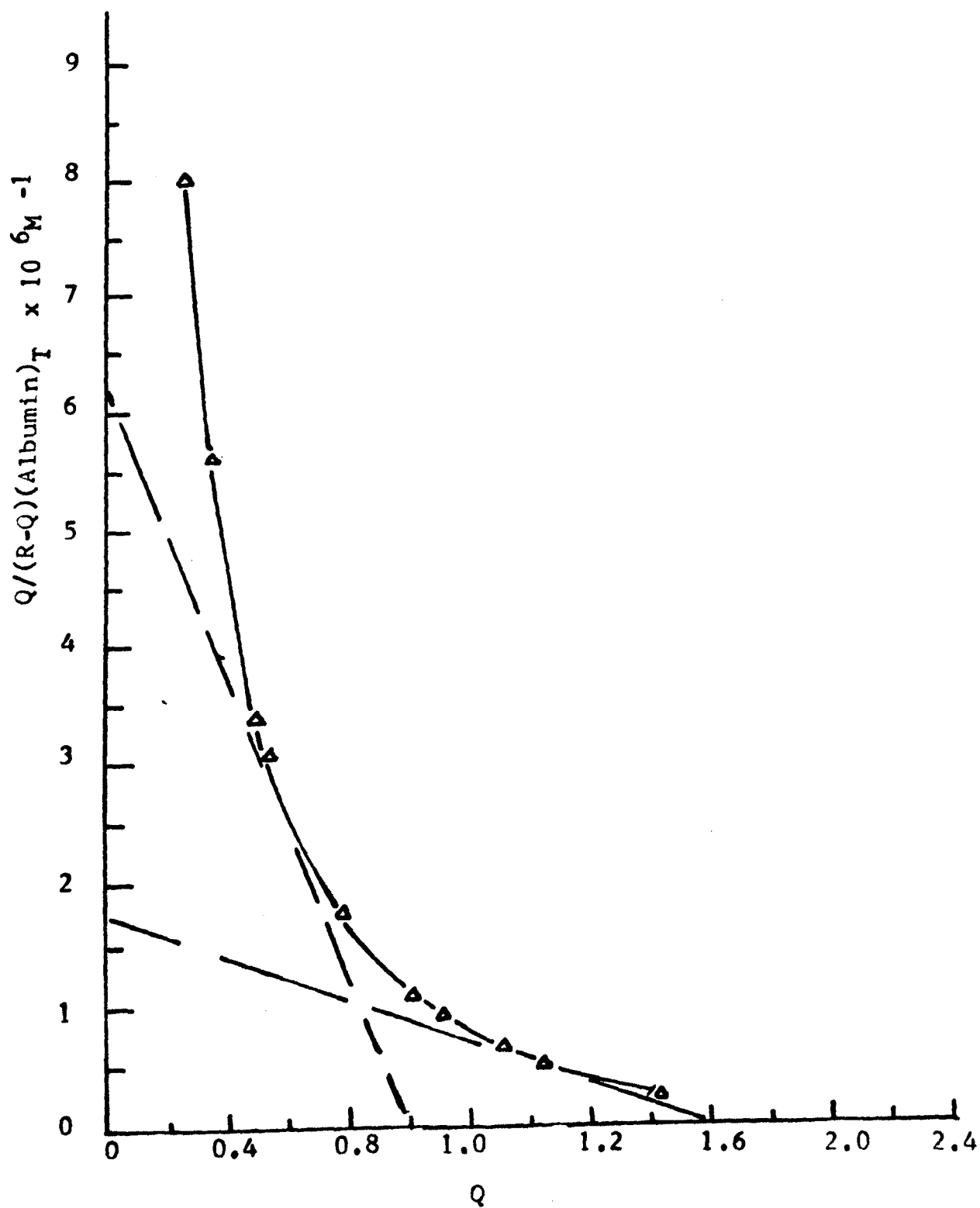
It appears that concentrations of albumin in the range of 10^{-5}M are not suitable for binding studies. The binding of mesobilirubin is accompanied by a loss in protein-intrinsic fluorescence. Because of the strong absorption of mesobilirubin in the region of protein emission, the most likely explanation of the quenching is resonance energy transfer from the excited tryptophan to mesobilirubin.

Calculations:

The Scatchard plot (Figure 34) shows that the human serum albumin has two association constants for mesobilirubin i.e. $K_1 = 6.15 \times 10^6 \text{M}^{-1}$ and $K_2 = 0.88 \times 10^6 \text{M}^{-1}$.

Fig.34. Scatchard plot of the binding of mesobilirubin to albumin in sodium phosphate buffer, pH 7.4, ionic strength 0.1M, at 24.0°C. Q is the average number of moles of mesobilirubin bound to albumin, and R is the ratio for maximal quench.

FIGURE 34.



The resulting calculations according to Levine²⁴⁴ are based on the following:

R is the molar ratio of mesobilirubin to albumin at maximal quench.

F_o is the fluorescence of albumin alone and F_1 is the fluorescence due to the quenching of albumin by mesobilirubin at a molar ratio of one.

The observed fluorescence, F, is given by the equation:

$$F = F_o - M, R$$

and

$$F_1 = F_o - M \text{ when } R = 1.0$$

The maximal quench is therefore given by

$$F_o - F_1 = M_1.$$

Q, is the average number of moles bound to albumin given by

$$F_o - F/m.$$

The high affinity binding of mesobilirubin ($K_a = 6.15 \times 10^6 M^{-1}$) compares very well with the weaker binding site of bilirubin ($K_a = 0.6 \times 10^6 M^{-1}$). Its weaker binding site is a magnitude of seven less than the high affinity site. The concentrations of albumin used was constant at $1.01 \times 10^{-6} M$ which was found to be in the region favourable for most fluorescence experiments.

Mesobilirubin, like bilirubin does not fluoresce in certain organic solvents and water. But when bound to albumin it shows some slight fluorescence. This extrinsic fluorescence is a measure of the bound mesobilirubin undergoing slow transformation. This change like that in bilirubin may involve a considerable entropy change. Studies at different temperatures would therefore be necessary to confirm this. During these studies it was observed by UV spectrophotometry that although

albumin/mesobilirubin complexes bind bilirubin, the albumin/bilirubin complexes did not. This is further confirmation that tighter binding occurs with bilirubin than with mesobilirubin. Table 4.2 shows the comparison of properties of mesobilirubin and bilirubin:

Table 4.2

Property	Mesobilirubin	Bilirubin ²⁴⁶
max in CHCl ₃ (nm)	435	453
in sodium phosphate buffer, pH 7.4, 0.1M	ca 412	438
with Albumin	438	460
Association Constants:		
k ₁	6.15 x 10 ⁶ M ⁻¹ +	7.0 x 10 ⁷ M ⁻¹
k ₂	0.88 x 10 ⁶ M ⁻¹	0.6 x 10 ⁷ M ⁻¹
Solubility:		
CHCl ₃	Yes	Yes
DMSO	Yes	Yes
0.2M Na ₂ CO ₃	Yes	Yes
Methanol	No	No
Na OH	Yes	Yes
Diazo reaction	Positive	Positive
Excretion	Conjugation	Conjugation

+ Measurements done by fluorescence at 24°C for Mesobilirubin and 25° for Bilirubin, pH 7.4.

Mesobilirubin is one of the intermediates in the catabolism of bilirubin to urobilin and stercobilin. Previous studies have shown the pigment to be found in the bile; and although its physiological role is yet

unknown, its appearance in the bile surely raises some questions. It may probably be that an enzyme system(s) exist(s) in the liver or kidney that reduces the vinyl groups to ethyl groups. The fact it is less tightly bound to albumin may imply a different carrier altogether. However, Goto (1956) showed that albumin binds mesobilirubin using paper electrophoresis. Despite all these observations all the "credit" went to bilirubin and investigators have since ignored the possibility that mesobilirubin could be as cytotoxic as bilirubin. This argument is supported by lack of bilirubin in deeply jaundiced individuals;⁶⁰ mesobilirubin was found as the kernicteric pigment in the basal ganglia of infants.⁸⁰ Many products of phototherapy in vivo are still unknown, it would be possible to speculate that some of these products are from mesobilirubin. Furthermore mesobilirubin was shown to produce photo-oxidation products such as methylethylmaleimide.¹⁵⁵ Indeed the synthesis of mesobilirubin is solvent dependent but in vivo synthesis could follow a different method altogether. Evidence exists as to the possibility of the same carrier in the plasma since labelled mesobilirubin and bilirubin were excreted at more or less the same time by normal rats. To circumvent this problem entails the improvisation of a method that would clearly distinguish mesobilirubin from bilirubin in the serum and the jaundiced sites.

Clinically, therefore resorption of mesobilirubin by the hepatic system could cause some displacement or competition at the weaker sites for bilirubin binding. However, since this phenomenon has not been recorded or observed, it is possible that some bile constituent quickly reduce mesobilirubin to other products. If, however, the weaker

binding site of bilirubin is the same as the high affinity for mesobilirubin, then the latter pigment could be used to study the topography of this site. If this speculation is correct it would serve the problem of using bilirubin which would not be a better probe for its secondary sites because of the existence of tighter binding sites.

The existence of mesobilirubin in the ganglia is obviously worth following. Unconjugated bilirubin not bound to albumin will not cross the brain-blood barrier, yet there have been suggestions that it may cross and then be reduced to mesobilirubin by the basal ganglia.⁵⁹ This is further complicated by lack of a clear definition of the blood-brain barrier. It is also possible that mesobilirubin existing in little quantities in the serum can cross the barrier. Although there is no supportive evidence to this; but it is known that anemia,²⁴⁷ anoxia,²⁴⁸ immaturity,²⁴⁸ hepatic toxins²⁴⁹ and the toxic effects of bile pigments²⁵⁰ are factors that may influence the permeability of the blood-brain barrier to a degree that would allow bilirubin to pass from the blood stream into the cerebral tissues. It was also shown that intact neurons take abundant mesobilirubin without the former exhibiting cytological changes,²⁵¹ but whether this is a short or long term effect is not known. This observation may be likened to the influence on the hepatic uptake of bilirubin by ligandin in the liver. It is possible that mesobilirubins by some yet unknown factor "drains" into the neurons which are likely to have a strong affinity for the pigment. The brain contains significant amount of sphingomyelin (SPH) with an association constant of $4.0 \times 10^6 \text{ M}^{-1}$ for bilirubin.²⁵¹ It is believed that this very high association of SPH (compared to other lipids) may allow tying up very low levels of unconjugated bilirubin

or weak binding bilirubin from serum albumin, or the so-called free bilirubin in patients with high serum concentration of unconjugated bilirubin. This may be one of the possibilities for bilirubin toxicity in the brain.²⁵ The toxicity becomes evident only when all the SPH molecules (in membrane as well) are tied up. It is necessary to recall that Vogel⁸⁰ observed that for a certain period mesobilirubin accumulated on to the neuron without causing any noticeable cytological changes. It is therefore possible that like in the case of SPH,²⁵² certain mesobilirubin binding factors within the neuron have to be saturated before any toxicity is evident.

Most of these phenomena are likely due to the strong association constant of specific lipids within the affected systems for the bile pigments. Since association constants of mesobilirubin with such lipids as SPH are not known it will suffice to speculate that from the little evidence available, mesobilirubin is a possible candidate for some of the neurological disorders currently attributed to bilirubin.

The binding studies of mesobilirubin have confirmed the importance of vinyl groups to the binding of albumin. A comparison of the high affinity association constants (Table 4.2) reveals that bilirubin is bound 11 times more strongly than mesobilirubin. If mesobilirubin is not as poisonous as bilirubin, then it is advantageous for the body to convert the latter pigment to the former.

4.4. Conclusion:

It goes without saying that the study of the binding of endogenous and exogenous substances to macromolecules will remain a field of interest to clinicians, pharmacologists and biochemists. Organic anions, like bilirubin and mesobilirubin can be used as probes to study certain phenomena likely to be exhibited by identical compounds within

the transport milieu. The results from the present studies of bilirubin can only afford a hypothetical explanation as to the binding site and the mechanism of phototherapy. More light can be expected from other approaches of mass spectra studies, kinetics of the formation of photoproducts of phototherapy, peptide mapping, analysis of the nature of intermediate products of phototherapy, etc. With an improved method of labelling bilirubin to high specific activity and consequently labelling the albumin, and the increasing task force of protein chemists now interested in bilirubin binding, it may not be long before the knowledge on the mechanism of bilirubin binding is complete. It is hoped that the present studies on mesobilirubin will further stimulate the need to study the chemistry and effect of this pigment whose mere similarity and derivation from bilirubin has militated against the study of its existence in the basal ganglia and serum.

Appendix I

Charcoal Treatment of HSA with some modifications:

The treatment was done according to Chen, R.F. (1967), 242,
J. Biol. Chem.¹⁷³

Albumin (10g) was dissolved in 100 mL of distilled water at 24°C. Charcoal Norit - A (5.0g) was added and the mixture lowered to pH 3.0 by adding 0.2M HCl. The solution was then placed on an ice bath and mixed magnetically for 1 hour. Charcoal was removed by centrifugation at 20,000 xg for 20 minutes in a Sorvall RC1 centrifuge with an 55 34 Rotor at 2°. The solution was further put on Sephadex G - 10 (0.6 x 60cm) and eluted with distilled water. This column was found to remove fines from charcoal which did not sediment during centrifugation. The clarified albumin was adjusted to pH 7.0 by addition of 0.2M NaOH

Source of Materials

Carboxymethy cellulose (CM)	Whetman Ltd., Maidstone, Kent.
Cyanogen bromide	Eastman Kodak Co., Rochester, N.Y.
Dansyl Amino Acids Standards	Sigma, London
Dansyl Chloride	Sigman, London
DEAE - cellulose	Whatman Ltd., Maidstone, Kent.
N-ethylmorphiline	B.D.H. Chemicals, Ltd., Poole, Dorset.
PM - 10 membranes	Amicon Corporation, Lexington, Mass., U.S.A.
Sephadex G-25	Pharmacia Fire Chemicals, Sweden.
Sephadex G-100	Pharmacia Fire Chemicals, Sweden.
Sephadex G-10	Pharmacia Fire Chemicals, Sweden
Sepharose 4B	Pharmacia Fire Chemicals, Sweden
Bilirubin	Sigman, London
Pepsin	Worthington Biochemicals, Freehold, New Jersey.
Polyamide Layer Sheets	E. Merck, Darmstadt.
Silica gel plates	E. Merck, Darmstadt.
Tritiated Water	E. Merck, Darmstadt.
H.S.A.	Sigma, London
10% Palladium/Charcoal	Johnson Matthey Chem. Ltd., London, England
n-Butyllithium	Aldrich Chem. Co., Gillingham, Dorset
Di-isopropylamine	Aldrich Chem. Co., Gillingham, Dorset
Hexamethylphosphoric triamide	Aldrich Chem Co., Gillingham, Dorset.

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Photoactivated Covalent Binding of [^3H]Bilirubin to Human Serum Albumin

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A one-step procedure has been developed for the preparation of [^3H]bilirubin IX- α in good yield from unlabelled bilirubin. Irradiation of an aqueous solution of [^3H]bilirubin IX- α in the presence of human serum albumin results in the covalent attachment of the bilirubin to the protein. Preliminary degradation studies have been carried out to locate the site of attachment of the bilirubin to the albumin.

Bilirubin is reversibly bound to serum albumin in plasma and is transported to the liver where it is conjugated and then excreted. Many experiments have been aimed at locating which domain of the albumin molecule binds bilirubin, but no conclusions have yet been made as to the exact site of binding. For example, affinity labelling of human albumin with bilirubin that had been activated with Woodward's reagent K (2-ethyl-5-*m*-sulphophenylisoxazolium hydroxide) (Gitzelmann-Cumarasamy *et al.*, 1976; Kuenzle *et al.*, 1976) indicates that two regions (residues 124–297 and residues 446–547) are attached to the bilirubin. However, objections can be raised to the activation of bilirubin by this reagent as the latter interacts initially with the carboxy groups of the bilirubin and for covalent attachment a suitably nucleophilic amino acid side chain of the albumin must be present in a favourable position in the binding site. Thus purely hydrophobic interactions between the albumin and the bilirubin are unlikely to be revealed by this method.

In the present paper we report the photoactivated covalent binding of [^3H]bilirubin to human serum albumin. This activation, which is brought about by light from a tungsten lamp, presumably involves free radicals and may not require the presence of nucleophilic groups in the binding site before a covalent attachment of the bilirubin to the albumin can occur. Thus this technique may reveal amino side chains that are involved in hydrophobic bonding to the bilirubin.

Materials and Methods

n-Butyl-lithium, di-isopropylamine and hexamethylphosphoric triamide were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K., and CNBr was obtained from Eastman Kodak Co., Kirkby, Liverpool, Lancs., U.K. Bilirubin IX- α and human serum albumin (fraction V) were obtained from Sigma (London) Chemical Co., Kingston upon

Thames, Surrey, U.K. The albumin was defatted before use by the method of Chen (1967).

Synthesis of [^3H]bilirubin

To a solution of di-isopropylamine (0.2 g, 2.0 mmol) in anhydrous tetrahydrofuran (80 ml) under an N_2 atmosphere was added *n*-butyl-lithium in hexane (1.0 ml, 1.6 M) and the mixture was stirred at -78°C for 30 min. Bilirubin (58.4 mg, 0.1 mmol) was added and the mixture was stirred at -78°C for 30 min after which hexamethylphosphoric triamide (1.5 ml, 8.55 mmol) was added. The mixture was stirred for a further 30 min and then trifluoro[^3H]acetic acid (0.546 ml, 4.8 mmol) was prepared by treating trifluoroacetic anhydride with an equimolar amount of $^3\text{H}_2\text{O}$ (5 Ci/ml; sp. radioactivity approx. 100 mCi/mmol). The reaction was stirred for 4 h at 21°C and then acidified with 0.1 M-HCl at 0°C . The aqueous solution was extracted with chloroform (3×100 ml), the chloroform extracts were combined, dried (Na_2SO_4) and evaporated to dryness *in vacuo*. The residual bilirubin was dissolved in chloroform (5 ml) and purified by preparative t.l.c. on silica gel with chloroform/methanol/water (40:9:1, by vol.) as solvent. The yellow band (R_f 0.75) was excised and eluted from the silica with chloroform. The chloroform was removed *in vacuo* and the residual bilirubin was crystallized from chloroform/methanol (1:1, v/v) to yield 19.5 mg. This bilirubin was then suspended in water and brought into solution by adding sufficient conc. aq. NH_3 (sp. gr. 0.88). After 5 min the solution was acidified with 1 mM-HCl and extracted with chloroform (100 ml). The chloroform was dried (Na_2SO_4) and evaporated *in vacuo* to give [^3H]bilirubin (19.3 mg, 33% yield from unlabelled material). The bilirubin ran as a single spot on t.l.c. (McDonagh & Assissi, 1971), had a light-absorption maximum in chloroform at 454 nm (ϵ 59 650 litre \cdot mol $^{-1}$ \cdot cm $^{-1}$) and had a sp. radioactivity of 1.3 mCi/mmol. Diazotization (Hutchinson *et al.*, 1972) of the [^3H]bilirubin

gave azopigments with at least 99% of the specific radioactivity of the bilirubin.

Covalent binding of [^3H]bilirubin to human serum albumin

A solution of [^3H]bilirubin ($65\mu\text{M}$) was prepared by dissolving 3.8mg (sp. radioactivity 1.3×10^9 c.p.m./mmol) in 0.2M-NaOH (0.2ml) and diluting to 100ml with 0.1M-sodium phosphate buffer, pH7.4. A mixture of this solution (5ml) and a solution of human serum albumin (5ml, $65\mu\text{M}$) in 0.1M-sodium phosphate buffer, pH7.4, was irradiated at 40cm from a tungsten lamp (150W) for 1h.

The reaction mixture was then applied to a column ($0.90\text{cm} \times 15.0\text{cm}$) of Sepharose-albumin (Kuenzle *et al.*, 1976). The column eluate was dialysed against 2 litres of 12.5mM-sodium salicylate, pH7.4, for 48h and then against 2 litres of 0.1M-HCl for 24h, after which time a non-diffusible bilirubin-albumin complex (5×10^7 c.p.m./mmol of albumin) was obtained. In a typical experiment, after irradiation for 1h a solution of albumin (5.0mg/ml) had an absorption of 0.50 at 450nm after chromatography on Sepharose-albumin. When the experiment was repeated with degassed solutions and with N_2 flushing during the irradiation, the residual specific radioactivity was virtually the same. If the irradiation step was omitted the specific radioactivity of the bilirubin-albumin complex was 2×10^6 c.p.m./mmol of albumin.

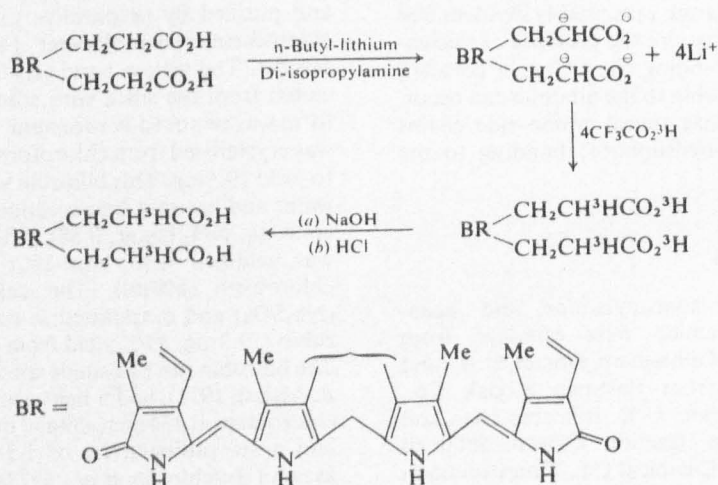
Degradation of bilirubin-albumin complex by CNBr

The bilirubin-albumin complex was dissolved in formic acid and degraded with CNBr (McMenamy

et al., 1971). After chromatography, three peptides were obtained [A (residues 298–585), B (residues 1–124) and C (residues 125–297)], the identities of the peptides being confirmed by the determination of their *N*-terminal amino acids. For the complex obtained after irradiation for 60min in the air, the maximum radioactivity (62%) was found in peptide B with lesser amounts in peptides C (32%) and A (3%). When the albumin obtained from the non-irradiated sample was degraded with CNBr no radioactivity could be detected in any of the peptides.

Discussion

Treatment of the lithium derivative of acetophenone with trifluoro[^3H]acetic acid has been described recently as a method for the synthesis of [$2\text{-}^3\text{H}$]acetophenone in high yield, the latter being used for the preparation of phenyl[$2\text{-}^3\text{H}$]glyoxal (Augustus & Hutchinson, 1979). We have now adapted this method for the one-step preparation of [^3H]bilirubin from unlabelled material. This method is preferable to the two-step procedure involving the preparation of bilirubin dimethyl ester (Hancock *et al.*, 1976) as the overall yield is higher and the specific radioactivity of the [^3H]bilirubin obtained is greater. By analogy with other reactions of metallated carboxylic acids (Creger, 1977), we assume that only one ^3H atom is introduced into each α -methylene group of the bilirubin. No ^3H is introduced into the bridge methylene group as diazotization of the [^3H]bilirubin yields azopigments that contain at least 99% of the initial radioactivity.



Irradiation of a mixture of [^3H]bilirubin together with one molar equivalent of human serum albumin with light from a tungsten lamp resulted in the bilirubin becoming firmly bound to the protein. Flushing the reaction mixture with N_2 before and during the irradiation did not have an appreciable effect on the amount of radioactivity that is firmly bound to the albumin. This suggests that either the binding is not an oxidative process, but may involve the addition of an amino acid side chain of the albumin to one or both of the *exo*-vinyl groups of the bilirubin (Manitto & Monti, 1972), or that trace amounts of O_2 remaining in the system are sufficient to allow the photo-oxidation of bilirubin to take place (Bonnett, 1976). The photo-activated bilirubin could then react with an amino acid side chain in the albumin molecule. The relative concentrations of albumin and bilirubin in these preliminary experiments were chosen so that high-affinity binding site in the albumin is just fully occupied. Thus it is hoped that complications due to the binding of bilirubin at high- and low-affinity sites do not arise to a significant extent.

We have found that it is difficult to remove final traces of radioactive bilirubin from the albumin even when no irradiation has taken place. A combination of chromatography on an affinity column of human serum albumin-Sepharose (Kuenzle *et al.*, 1976) and exhaustive dialysis against sodium salicylate and 0.1 M-HCl failed to remove the final traces of radioactivity. However, the covalent attachment of bilirubin to the albumin after irradiation could be clearly demonstrated by degradation of the latter with CNBr (McMenamy *et al.*, 1971). No radioactivity was associated with the degradation products unless irradiation of the bilirubin/albumin mixture had taken place.

The majority of the radioactivity (62%) is associated with the peptide corresponding to residues 1–124 and 32% was associated with residues 125–297. This contrasts with the observations of Gitzelmann-Cumarasamy *et al.* (1976), who found that two regions in the albumin (residues 124–297 and 446–547) become bound to the bilirubin. This difference may be due to the difference in manner in which the bilirubin is activated. After activation with Wood-

ward's reagent K, the carboxy groups of the bilirubin may be unable to react with any amino acid side chains in the binding site, but may react with nucleophilic groups that are remote from this site. Alternatively, in our experiments the *Z,Z*-isomer of bilirubin IX- α may undergo a configurational change during irradiation to one of the three other geometric isomers of bilirubin IX- α (McDonagh *et al.*, 1979) before reaction with the albumin takes place. We have been unable to detect chromatographically the formation of other isomers of bilirubin, e.g. the III- α or XIII- α isomers, during our irradiation experiments. It is relevant that Geisow & Beaven (1977) found that bilirubin bound non-covalently to two albumin-derived peptides corresponding to residues 1–386 and 49–307 respectively.

Further experiments are required to investigate the nature of the binding reaction and to locate exactly the site of attachment of the bilirubin to the albumin.

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